DISSERTATION

Biological control of clubroot (*Plasmodiophora brassicae*) by an endophytic fungus (*Acremonium alternatum*)

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Abstract

The biological control of plant pests with beneficial microbes has become increasingly important over the last decades. Soil microbes such as fungi and bacteria colonise the roots of plants and promote their growth. Some beneficial microbes can trigger a weak plant defence response that enhances the immune response of the plant at subsequent pathogen attacks and therefore increase the resistance of the plant to other invaders. This mechanism is called "priming".

While biocontrol agents are applied against a variety of plant pests fundamental knowledge of the molecular mechanisms of plant-microbe interactions is still lacking. Especially molecular studies on the role of resistance genes in the interaction of plants with beneficial endophytic fungi are rare.

In this study it was investigated how the fungal biocontrol agent *Acremonium alternatum* affects the development of the clubroot pathogen *Plasmodiophora brassicae* within the plant host *Arabidopsis thaliana*. Clubroot is a devastating disease in crop plants such as cabbage and rapeseed and causes abnormal root growth that leads to so called "club roots". *P. brassicae* develops within the plant roots and forms resting spores that are very durable and stay infective in soils for up to 2 decades. The control of clubroot by chemical means is difficult and the disease continues to spread on all continents and was also found in Saxony, Germany in recent years.

In 2 preliminary studies the co-inoculation of clubroot plants with the fungus *A. alternatum* resulted in reduced clubroot symptoms in Chinese cabbage and Arabidopsis. It was therefore hypothesised that *A. alternatum* induces resistance mechanisms in the plant and thus enhances immunity.

The focus of this study was to test this hypothesis by carrying out expression analyses on root tissue of infected Arabidopsis plants. For this the plants were inoculated with spores of *P. brassicae* and *A. alternatum* before RNA was extracted from the roots, followed by cDNA synthesis and quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR). A microarray of root tissue of infected Arabidopsis plants was carried out to depict the events at the stage of initial root hair infection with the clubroot pathogen. The findings from the gene expression analyses were verified for 2 genes with Arabidopsis mutants that are defective in the respective gene and with 2 overexpressor lines.

Clubroot symptoms were assessed by rating the root galls according to their stage of

development. The overall plant health was further evaluated by recording the developmental stage of the plants (generative vs. vegetative), stem lengths and plant biomass. In addition, 2 local varieties of the economically important crop plant rapeseed (*Brassica napus* var. Ability and var. Visby) were investigated with qRT-PCR and by recording the disease parameters just described.

A second goal of this study was to assess the general biocontrol potential of the yet relatively unknown endophyte *A. alternatum* in terms of enzymatic activity and competitive behaviour against other phytopathogenic fungi. The potential of this fungus for the use in integrative pest management was investigated. The results presented here are novel findings for this fungus and have not been studied before.

The microarray from Arabidopsis roots revealed that the clubroot pathogen *P. brassicae* suppresses its recognition by pathogen receptors of the plant and thus prevents the host to induce resistance mechanisms. The fungus *A. alternatum* boosted the level of the pathogen recognition-related genes *BAK1* and *FLS2* and thus helped to establish early plant defence responses. PCR analyses confirmed that these early responses led to salicylic acid-dependent resistance in the plants which was maintained for several days as shown by elevated levels of the *PATHOGENESIS-RELATED* gene *PR1*. Marker genes for an alternative resistance pathway that is mediated over the plant signals jasmonate and ethylene were not activated in Arabidopsis.

The co-inoculation of Arabidopsis plants with the endophyte *A. alternatum* resulted in a significant reduction of clubroot symptoms by up to 24%. In rapeseed the reduction of disease symptoms was 19% and 28% when the plants were treated with a crude cell wall extract of *A. alternatum* before inoculation with the clubroot pathogen. PCR analyses from Arabidopsis showed a strong response of pathogen recognition genes to the cell wall extract and spores of the endophytic fungus. In rapeseed all of the investigated pathogen recognition genes were upregulated after the endophyte treatment but not with the clubroot pathogen. Together with the PCR results from the microarray these findings suggest that *A. alternatum* primes its host plant and enhances the resistance of the plant towards *P. brassicae*. In addition, the fungus increased biomass, stem lengths and survival rates of clubroot-infected plants.

In vitro tests revealed that the endophyte can solubilise phosphate and is not very competitive against other phytopathogenic fungi such as *Aspergillus* or *Fusarium* which is likely an effect of the relatively slow growth of the endophyte on agar plates.

From this study it can be concluded that i) the fungus *Acremonium alternatum* induces resistance mechanisms in Arabidopsis and 2 *Brassica napus* cultivars and facilitates the recognition of the clubroot pathogen *Plasmodiophora brassicae*; ii) that Arabidopsis and *Brassica* react differently to this beneficial microbe, a fact that has been observed for *Plasmodiophora* and other microorganisms as well; iii) living spores are not necessary for clubroot biocontrol in rapeseed as a crude cell wall extract reduces symptoms more

efficiently.

Overall the endophyte *A. alternatum* is a very promising candidate for the use in integrative pest management in plant strengtheners or as biocontrol agent.

Zusammenfassung

Die biologische Kontrolle von Pflanzenkrankheiten gewinnt zunehmend an Bedeutung. Bodenbewohnende Mikroben wie Pilze oder Bakterien kolonisieren die Wurzeln von Pflanzen und fördern deren Wachstum. Einige dieser förderlichen Mikroben aktivieren eine schwache Abwehrreaktion in der Pflanze die sich verstärkt bei einer weiteren Infektion mit einem Krankheitserreger. Dieser Mechanismus, den man "Priming" nennt, führt zu einer verbesserten Resistenz der Pflanze gegenüber Pflanzenpathogenen.

Obwohl natürliche Schädlingsbekämpfer bereits gegen eine Vielzahl an Krankheiten eingesetzt werden, weiss man über grundsätzliche molekulare Mechanismen dieser Pflanzen-Mikroben-Interaktionen nur wenig. Besonders die Rolle von Resistenzgenen ist bisher wenig erforscht, welche bei der Beziehung zwischen Pilzen und Pflanzen eine Rolle spielen.

In der hier vorliegenden Arbeit wurde untersucht, wie der endophytische Pilz *Acremonium alternatum* die Entwicklung des Krankheitserregers *Plasmodiophora brassicae* in der Pflanze *Arabidopsis thaliana* beeinflusst. Die Kohlhernie, ausgelöst von *P. brassicae*, ist eine verheerende Krankheit die u. a. bei Kohl und Raps auftritt und Wurzelgallen, so genannte "Hernien", hervorruft. Der Krankheitserreger entwickelt sich im Wurzelsystem der Pflanze und bildet Dauersporen, die bis zu 20 Jahre lang im Boden infektiös überdauern können.

Ein Eindämmen der Krankheit mit Pflanzenschutzmitteln ist durch den komplexen Lebenslauf des Erregers sehr schwierig, das führte zu einer weltweiten Verbreitung der Kohlhernie. Auch in Sachsen wurden in den letzten Jahren Fälle von Kohlhernie gemeldet.

Wie 2 Studien zeigen, führt die Ko-Inokulation von Kohlhernie-erkrankten Pflanzen mit *A. alternatum* zu einer Verringerung der Symptome in Chinakohl und Arabidopsis. Es wurde daher die Hypothese aufgestellt, dass der Pilz Resistenzmechanismen in der Pflanze anschaltet und damit ihre Immunität erhöht.

Um diese Hypothese zu testen, wurden in der hier vorliegenden Studie Genexpressionsanalysen an infizierten Arabidopsiswurzeln durchgeführt. Dafür wurden die Pflanzen zunächst mit Sporen des Kohlhernieerregers und des Pilzes inokuliert, es wurde RNA aus den Wurzeln extrahiert, in cDNA umgeschrieben und diese mittels quantitativer Reverse-Transkriptase-Polymerasenkettenreaktion (RT-qPCR) untersucht. Ein Microarray von Wurzeln infizierter Pflanzen wurde durchgeführt um die Ereignisse abzubilden, die sich zeitnah nach der Infektion in den Wurzeln abspielen.

Die Ergebnisse der Genexpressionsanalysen wurden dann an Arabidopsismutanten, die einen Gendefekt im jeweiligen Gen haben, und an Überexprimierer-Pflanzen verifiziert.

Kohlherniesymptome an Pflanzen wurden durch eine Kategorisierung der Schadsymptome erfasst. Die allgemeine Pflanzengesundheit sowie der Entwicklungsstand der Pflanze, Stengellängen und das Frischgewicht wurden bestimmt.

Zusätzlich wurden 2 Rapssorten, die in Sachsen angebaut werden, untersucht im Hinblick auf die Krankheitsenwicklung und die Reguation von Abwehrgenen.

Ein weiteres Ziel dieser Arbeit war es das Biokontrollpotential des bisher schlecht untersuchten Pilzes *A. alternatum* zu bestimmen. Dazu wurde *in vitro* die Enzymaktivität des Pilzes getestet sowie seine Konkurrenzfähigkeit gegenüber anderen pflanzenpathogenen Pilzen. Das Potential des Pilzes für die Anwendung im integrierten Pflanzenschutz wurde getestet. Die hier präsentieren Ergebnisse stellen neue Erkenntnisse dar, die für diesen Pilz noch nie untersucht wurden.

Der Microarray von Arabidopsiswurzeln zeigte, dass der Kohlhernieerregers die Erkennung durch die Pflanze verhindert und damit Abwehrmechanismen verhindert. Der Pilz *A. alternatum* förderte die Aktivität der pflanzlichen Erkennungsrezeptoren FLS2 und BAK1 und setzte damit die Erkennung von *P. brassicae* in Gang. PCR-Analysen ergaben, dass diese früh induzierten Abwehrmechanismen zu einer systemischen Resistenz in der Pflanze führte durch die Aktivierung des Pathogenese-relevanten Gens *PR1*. Genmarker, die die Aktivität eines alternativen, von Jasmonat und Ethylen vermittelten Abwehrweges anzeigen, waren nicht ativiert.

Die Ko-Inokulation von Arabidopsis mit dem Endophyten führte zu einer signifikanten Reduktion der Krankheitssymptome um 24%. In Raps betrug die Reduktion 19% und 24% wenn die Pflanzen vor der Kohlhernie-Infektion mit einem Zellwandextrakt des Pilzes behandelt wurden. Mittels PCR konnte gezeigt werden, dass Gene für das Erkennen von Pathogenen in der Wurzel von Arabidopsis auf den Zellwandextrakt und Sporen des Pilzes reagieren. In Raps wurden alle der untersuchten Erkennungsgene aufreguliert nach der Infektion mit *A. alternatum*, nicht jedoch bei der Infektion mit *P. brassicae*.

Zusammenfassend lässt sich sagen, dass der endophytische Pilz *A. alternatum* die Wirtspflanze auf eine folgende Infektion vorbereitet (Priming) und systemische Abwehrmechanismen in der Pflanze induziert, wenn diese mit Kohlhernie infiziert ist. Außerdem treibt der Pilz das Sprosswachstum voran, erhöht die Biomasse und fördert das Überleben von Kohlhernie-infizierten Pflanzen.

In vitro-Tests ergaben, dass der Endophyt Kalziumphosphat löslich machen kann und wenig kompetitiv gegenüber Pflanzenpathogenen wie *Aspergillus* oder *Fusarium* ist. Dies ist vermutlich mit dem langsameren Wachstum des Endophyten im Gegensatz zu den anderen Pilzen zu erklären.

Aus den Ergebnissen dieser Arbeit lassen sich folgende Schlüsse ziehen: i) der en-

dophytische Pilz *Acremonium alternatum* induziert Resistenzmechanismen in Arabidopsis und Raps und und fördert die Erkennung des Kohlhernieerregers *Plasmodiophora brassicae*; ii) Arabidopsis und Raps reagieren unterschiedlich auf diesen förderlichen Pilz, ein solcher Unterschied wurde bereits für *Plasmodiophora* und andere Mikroben beschrieben; iii) lebende Sporen des Pilzes sind nicht notwendig um Krankheitssymptome der Kohlhernie in Raps zu verringern, ein Zellwandextrakt von *A. alternatum* ist dafür besser geeignet.

Ganz allgemein lässt sich sagen, dass der endophytische Pilz *Acremonium alternatum* ein sehr vielversprechender Kandidat ist für den Einsatz im integrierten Pflanzenschutz in Pflanzenstärkungsmitteln oder als Biokontrollorganismus.

1. Introduction

1.1. The clubroot disease - a worldwide threat to Brassica crops

Plasmodiophora brassicae is the causal agent of the clubroot disease. This soil pathogen causes one of the most damaging diseases within the family Brassicaceae (Voorrips 1995; Dixon 2009). It infects a wide range of crop plants, e.g. *Brassica napus, B. oleracea, B. pekinensis* and *B. rapa*, and can also establish pathogenicity in *Arabidopsis thaliana* (Fuchs and Sacristán 1996; Kobelt et al. 2000). Clubroot-infected plants are dwarfed compared to healthy plants and their roots grow abnormally large into galls. This root phenotype is called club, hence the name clubroot. At maturity, the galls turn brown and a large portion of the infected roots remain under ground when the plants are harvested. Thereby the spores are liberated from the plant tissue and remain infectious in the soil for 18 years or more (Wallenhammar 2008).

As *P. brassicae* also infects non-crop plants that belong to the Brassicaeae, fields that contain spores of the pathogen are generally not suitable anymore for the cultivation of cabbage.

1.1.1. The life cycle of P. brassicae

The life cycle of the clubroot pathogen is very complex and consists of 2 phases. The primary phase is restricted to root hairs and epidermal cells of the host and the secondary phase occurs in the cortex and stele of roots and hypocotyls and leads to the abnormal root development (Ingram and Tommerup 1972). During the second phase the pathogen develops from a dikaryotic amoeba-like structure to large multinucleate plasmodia which are present within the host cell. In a late state of infection the plasmodia form resting spores which are liberated into the soil when the host tissue decays (Ludwig-Müller 1999; Fig. 1.1).

1.1.2. Physiological and molecular characterisation of the clubroot infection

In early stages of the infection the pathogen uses plant signaling molecules to re-distribute assimilates from the shoot to the root to guarantee its own nutrition (Evans and Scholes

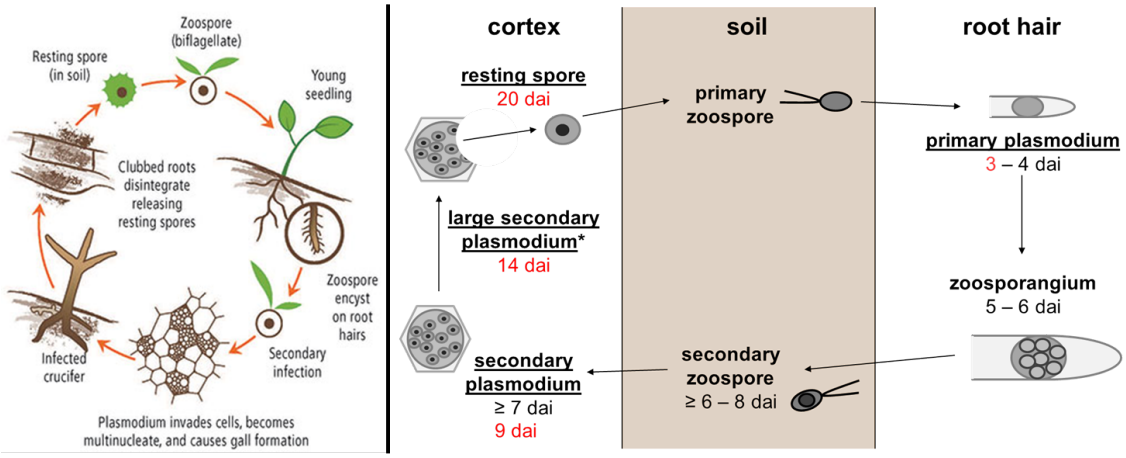


Figure 1.1.: The life cycle of *Plasmodiophora brassicae*. Left is an overview (source: Ohio State University), right the life cycle in more detail according to Ingram and Tommerup (1972); Kobelt et al. (2000) and Kageyama and Asano (2009). In red are investigated time points from this study. Abbreviations: dai - days after inoculation.

1995; Gravot et al. 2012). This leads to a shortage of nutrients in the upper plant parts in later stages because photosynthesis cannot be sustained anymore, leading to withering and finally death of the host plant (Ludwig-Müller 1999).

So far it remains mostly unclear which signals the pathogen is using to reprogram its host. Roughly one hundred genes had been identified from *P. brassicae* until 2006 but for most of them no function was assigned due to the low homology of sequences to other organisms (Bulman et al. 2006). Just recently the genome of *P. brassicae* was sequenced by 2 independent research groups but annotation of genes is still going on (Schwelm et al. 2015).

Siemens et al. (2009) found marker genes (*PbActin* and *PbCC243*) for the presence of the pathogen during infection in Arabidopsis ecotype Col-0. They also found genes that can be attributed to specific stages within the life cycle of *P. brassicae*: *PbBrip9* and *PbCC24* were expressed during late developmental stages of the pathogen, correlating with the increase in sporulating plasmodia. Generally, detection of most *P. brassicae* genes correlated with the increasing number of secondary plasmodia colonising the root, starting 14 days after infection (dai). At an earlier point of time (7 dai) for most of the investigated 13 pathogen transcripts no or an extremely low expression was found (Siemens et al. 2009).

A serine protease gene (*PRO1*) was identified during the primary infection stage that was connected to the stimulation of resting spore germination of *P. brassicae* (Feng et al. 2010).

Ludwig-Müller et al. (2015) found a methyltransferase gene (*PbBSMT*) that is similar to plant methyltransferases and methylates salicylic acid to the inactive form methyl salicylate at late stages of the infection (28 dai in Arabidopsis).

In a compatible interaction, signs of induced defence in the host root hair have been

reported as deposition of callose between the host plasma membrane and the cell wall at the penetration site of the pathogen (Aist and Williams 1971).

For the second infection cycle no evidence for an induced defence reaction such as necrotic responses was found, reflecting the high degree of compatibility between host and parasite (Fuchs and Sacristán 1996). Growth hormones like cytokinins and auxins are known to be involved in symptom development (Devos et al. 2006; Siemens et al. 2006; Ludwig-Müller and Schuller 2008; Diederichsen et al. 2014).

The pathogen produces small amounts of cytokinin at the beginning of the infection (Müller and Hilgenberg 1986) and at the same time inhibits the cytokinin-oxidase of the plant (Siemens et al. 2006). Elevated cytokinin levels lead to prolonged cell divisions and cell elongations in infected roots and hyptocotyls and an increased photosynthetic rate in the leaves. At the same time, the gall is established as a sink tissue for photosynthetic products (Ingram and Tommerup 1972, Evans and Scholes 1995, Ludwig-Müller et al. 2009). The enhanced development of sporangia causes a drastic increase of the cellular volume, called hypertrophy. Here, as well as for cell division, an elevated auxin pool is necessary and has been found (Ludwig-Müller et al. 1999, Devos et al. 2005). Concomitantly, genes and enzymatic activities connected to indole-3-acetic acid (IAA, auxin) biosynthesis are elevated (reviewed in Ludwig-Müller and Schuller 2008).

Also several glucosinolates were found to be upregulated in root galls and a positive correlation between indole glucosinolate content of the galls and disease severity was reported (Ludwig-Müller 2009). Glucosinolates are secondary metabolites of the Brassicaceae family with antimicrobial properties against biotroph pathogens in particular and their biosynthesis is connected to the biosynthesis of IAA (Ludwig-Müller 2009; Pieterse et al. 2012).

1.1.3. Problems in controlling clubroot

The clubroot disease is difficult to control by either chemical or cultural means since there is no potent agrochemical available that is acceptable on an economical and ecological basis (Donald et al. 2009; Diederichsen et al. 2014).

An alkaline pH provides unfavourable conditions for clubroot development and has been shown to decrease symptoms (Donald et al. 2009). However, consistent liming is not a suitable strategy to eliminate the pathogen. On the one hand it leads to deterioration of the soil by accelerating top soil degradation and advances boron deprivation in periods of drought. On the other hand crop rotation, which is an essential tool to prevent the spread of most soil borne diseases, cannot be applied under constant alkaline conditions. When the spore density is high enough and other conditions such as soil moisture and temperature are suitable, the disease develops independently from the pH value (Gossen et al. 2014).

Breeding programs have yielded resistant cultivars that can be grown for a few years on

the field before the pathogen evolves more virulent pathotypes on these plants (Diederichsen and Sacristan 1996; Diederichsen et al. 2009). Up to now control of *P. brassicae* is only possible with complex methods that are exercised in Australia (Donald et al. 2009). Due to an extensive spread of the pathogen in the 1980s and 1990s Australian farmers suffered high yield losses during that period and a coordinated national program was started with the aim to reduce crop losses (Donald and Porter 2014). Still, clubroot can be found now on soils of all major production regions in Australia which makes a constant monitoring and control of the disease a top priority.

In Canada immense investment is put into the control of clubroot since an outbreak in central Canada in 2003 and the continous spread of the disease to neighbouring provinces since then (Hwang et al. 2014). Here farmers rely heavily on resistant cultivars (Cao et al. 2014). However, since a breakdown in resistance has been observed in the past after a few growing periods, the farming of resistant cultivars cannot be applied as single strategy and does not eliminate the pathogen from the soil (Hwang et al. 2014).

Manipulating auxin and cytokinin levels has been shown to cause tolerance to clubroot, however, interfering with the hormonal balance of the plant causes growth defects which are undesirable, such as development of adventitious and lateral roots, inhibition of leaf abscission, promoted growth of axillary buds and early senescence. This could be partially circumvented by using root specific promoters but the generation of genetically modified organisms is not always acceptable in practice. Therefore, alternative strategies are needed. One possibility would be to boost the plant defence system by biocontrol organisms.

Microarray data showed that a large part of defence genes in Arabidopsis roots are either not or even down-regulated during infection with *P. brassicae* (Siemens et al. 2006; Agarwal et al. 2011). Agarwal et al. (2011) concluded from their microarray that the biosynthesis of salicylic acid and ethylene is repressed by the pathogen whereas biosynthesis of jasmonic acid is possibly induced during early stages in the infection. Newer findings emphasise the role of a clubroot methyltransferase ithat methylates the salicylic acid signal and thus manipulates plant defence (Ludwig-Müller et al. 2015).

It was therefore hypothesised that *P. brassicae* is able to suppress the defence strategy of the host plant. In consequence, the induction of resistance responses could be used to control clubroot development. This could be achieved by treatment with chemicals or plant endophytes, the latter occurring naturally in the soil. This concept has only to a minor part been investigated so far with the clubroot pathogen.

It is known that soilborne microbes such as fluorescing pseudomonads or endophytes such as arbuscular mycorrhizal fungi and the growth promoting fungus *Piriformospora indic*a are able to increase the plant's tolerance to pathogens (Waller et al. 2005). The induction of induced systemic resistance caused by bacteria is mediated by plant signals such as jasmonic acid and ethylene, in contrast to the systemic acquired resistance which

is mediated by salicylic acid (Pieterse and van Loon 2004, see also section 1.4). It is less clear, however, how the resistance or tolerance to pathogens is induced by beneficial fungi, although evidence was provided for the involvement of jasmonic acid and *NPR1* in systemic resistance induced by *P. indica* to powdery mildew in Arabidopsis (Stein et al. 2008, Lahlali et al. 2014). These examples show that endophytic fungi are in principle able to induce defence mechanisms in plants. Indications that this principle might be applicable to clubroot control are described below.

1.1.4. Resistance to clubroot

Since farmers are interested in clubroot resistant crops research focused mainly on crop plants such as *Brassica napus, B. oleracea* and *B. rapa* for resistance breeding (Diederichsen et al. 2009; Piao et al. 2009). The problem here is, that most of the resistance genes found so far in *Brassica* crops are race-specific.

Resistance tests against clubroot can suffer from a considerable amount of environmental variation as weather and soil conditions on the field are not constant over the whole growing period of crops (Voorrips 1992). Therefore, Arabidopsis has been used to develop resistance markers, i. e. quantitative traits to measure the extent of the clubroot disease, because the size and short generation cycle of this model plant allows for testing with more plants under constant conditions than it would be possible with crop plants like cabbage or rapeseed (Siemens et al. 2002).

The role of aliphatic and indole glucosinolates as defence compounds or simply auxin precursors has been discussed in the context of plant defence (Ludwig-Müller 2009; Ludwig-Müller et al. 2009). It is assumed that the degradation of glucosinolates via myrosinase and other proteins lead to metabolites that act as defence compounds against clubroot in Arabidopsis to some extent (Ludwig-Müller 2009). However, the interplay between gluscosinolates, auxin and plant defence is not fully understood yet.

In recent years, studies have focused rather on the changes in hormone metabolism in roots infected with *P. brassicae* (e. g. De Vos et al. 2005; Ando et al. 2005; Devos et al. 2006; Siemens et al. 2006; Ludwig-Müller et al. 2009; Schuller et al. 2014) than on the molecular basis of quantitative resistance for which only few studies were available until the beginning of this project in 2009 (Siemens et al. 2006). With an increasing number of microarrays and genetic studies in Arabidopsis in recent years our knowledge about the pathogen has greatly increased (Agarwal et al. 2011; Jubault et al. 2013; Lahlali et al. 2014).

In this study, the the model plant Arabidopsis and 2 German rapeseed cultivars were used to identify resistance genes which show a response to the inoculation with *P. brassicae* and the co-inoculation with a beneficial fungus.

1.2. A. alternatum reduces disease symptoms caused by P. brassicae

Endophytic fungi which live inside the host tissue and do not cause any signs of disease are present in almost all higher plants (Azevedo et al. 2000). Many *Acremonium* species protect their host from fungal pathogens and other diseases (Azevedo et al. 2000; Li 2008; Jäschke et al. 2010). Our group recently found that co-inoculation of *Arabidopsis thaliana* and *Brassica rapa* with *A. alternatum* resulted in delayed development of *P. brassicae* (Doan et al. 2010).

A. alternatum is a soil-borne and widespread fungus (Gams 1971) (Ascomycotina, Clavicipitacea) that is transmitted horizontally by spores and does not cause any symptoms of disease, nor does it alter the growth of its host plants (Raps 1997).

The beneficial effect of *A. alternatum* on various host plant – pathogen or pest interactions has been demonstrated (Raps and Vidal 1998; Romero et al. 2003; Kasselaki et al. 2006; Jallow et al. 2008; see also section 1.1). *A. alternatum* was able to reduce infection by powdery mildew (*Leveillula taurica*) on tomato plants (Kasselaki et al. 2006) and by *Sphaerotheca fusca* in melon (Romero et al. 2003). It also colonises Arabidopsis readily as shown in recent studies (Li 2008; Jäschke et al. 2010; Doan et al. 2010). This makes it a useful tool to investigate general interactions of the fungus with its host plant because genes involved in the interaction can be much easier assessed in the well-studied Arabidopsis than in *Brassica* sp. However, several studies have shown that differences exist between *Brassica* species and the model plant Arabidopsis in terms of gene regulation and enzymatic activity during the control of gall development (reviewed in Ludwig-Müller 2009).

In previous studies 14-day-old Arabidopsis Col-0 and *B. rapa ssp. pekinensis* were infected with *P. brassicae* by injecting the soil around each plant with a resting spore suspension of the pathogen (Jäschke et al. 2010; Doan et al. 2010). *A. alternatum* spores or extracts were administered likewise at different time points before or after inoculation with *P. brassicae*. Clubroot-infected plants inoculated with *A. alternatum* showed less disease symptoms under long-day conditions than plants infected with *P. brassicae* only. They had smaller galls with only slight hypertrophy of the roots and a higher aerial biomass production.

In addition, 2 parameters to quantify the disease - disease index and infection rate - were reduced. The disease index uses a scale of 5 classes to quantify disease symptoms and was therefore used as a measure for the effect of *A. alternatum* on *P. brassicae* infection (Klewer et al. 2001; Jäschke et al. 2010). A low disease index, i. e. smaller galls, correlated with smaller amounts of *P. brassicae* actin transcript. Regarding the suppression of pathogen development the best results were obtained when the 2 organisms were administered at the same time. Multiple inoculations with *A. alternatum* prior to *P.*

brassicae infection had no effect.

In *B. rapa* the disease index was reduced by 40%. The infection rate was not so much affected in *A. thaliana* showing once more the necessity to investigate both *Brassica* and Arabidopsis. Inoculation with 10^7spores of *A. alternatum* per ml per plant was sufficient to establish tolerance in Arabidopsis against the clubroot pathogen (Jäschke et al. 2010).

Differences in development of *P. brassicae* were found to be distinctive in mature galls (Jäschke et al. 2010). Mature galls without co-inoculation contained either plasmodia or sporulating plasmodia in most samples. In senescing gall parts the main developmental stages of the pathogen were resting spores. In mature galls co-inoculated with *P. brassicae* and *A. alternatum* only plasmodia were found. Within the senescing parts of these galls sporulating plasmodia were present that represent the onset of resting spore formation; however these resting spores were not yet fully developed. In infected roots that did not display clubroot symptoms smaller and less numerous plasmodia were present. So far its not clear whether the observed delay in resting spore formation is simply a competition about nutrients and / or space during infection or whether the defence responses of the host plant might be induced by either live spores ore signalling molecules of the fungus which could be also spore walls or fragments.

Expression analyses of genes from the protist *P. brassicae* during the infection revealed that *A. alternatum* caused a delay in regulation of most of the 12 pathogen genes, which are differentially expressed during the disease progress (Siemens et al. 2009). A germination assay showed that *P. brassicae* resting spore germination was not inhibited by *A. alternatum* spores or spore extracts. In addition, zoospore movement of the pathogen was also observed (Jäschke et al. 2010). Therefore, the most likely point of interaction is the entry into the host root.

Until the starting point of this study, no expression analyses of resistance genes had been done in this interaction.

1.3. The endophytic fungus Acremonium alternatum

The filamentous anamorphic fungus *A. alternatum* was first described in Gams´work on "Cephalosporium containing fungi" (1971). It belongs to the Clavicipitaceae (Hypocreales; Ascomycotina). *A. alternatum* is the lectotype of the genus *Acremonium* and weakly associated with the sister species *A. kiliense* and *Emericellopsis minima* (18S rDNA; Glenn 1996). Summerbell et al. (2011) presented a revised nomenclatural system of all acremonioid fungi based on ribosomal small and large subunit sequences (SSU/LSU). They confirmed the status of *A. alternatum* within the Hypocreales and identified the culture CBS 407.66 as holotype (deposited in Rostock, Germany).

The fungus has thin, septate hyphae and conidia in chains or heads. Colonies on agar plates are compact with abundant aerial mycelium and a yellowish to rose-orange pig-

mentation after exposure to light. The fungus grows relatively slow on PDA medium and prefers higher (26 - 28° C) to lower temperatures like 20° C (S. Auer, unpublished results). This is in accordance with the reported presence of the fungus in the mediterranean area of Europe, where soil temperatures easily reach 25° C and more (Malathrakis 1985).

Generally, Acremonium species and can be found in tropical and temperate regions (Glenn 1996) and are commonly isolated from asymptotic monocots such as rice (Ramanan et al. 1996).

1.3.1. A short history of A. alternatum as biological control agent

A. alternatum is a grass endophyte (Glenn 1996) that forms a tight bond with its host and can be transmitted asexually within the seeds of a host plant to the next generation (Raps and Vidal 1998). Generally these kind of endophytes are mutualists that can improve the competitive ability of their host and make it more tolerant to abiotic stresses (Clay 1993). Up to now the fungus is not known to produce antimicrobial compounds such as alkaloids (Raps and Vidal 1998).

Literature from the last 2 decades mention this fungus in connection with the suppression of several plant diseases (see Table 1.1).

The first paper in this context is from 1996 where *A. alternatum* was used against tar spot on coconut (Bettiol 1996). The fungus was isolated as mycoparasite from stromas of *Coccostroma palmicola* and *Catacauma torrendiella* in a commercial orchard. A fungal spore suspension (10^5 propagules/ml) of *A. alternatum* was sprayed on cultivated areas and on the coconut trees. This treatment was 5 times more effective and cost efficient than the use of a fungicide (Bettiol 1996). Today, however, this species is not in use anymore, instead South American farmers shifted to the sister species *A. cavaraeanum* which has been shown to have antimicrobial activity (W. Bettiol and D. Warwick, pers. communication).

By accident, researchers from Crete came across the endophyte when they studied powdery mildew in cucurbits (Malathrakis 1985). They isolated the species from cucurbit leaves that had been mildew infected and hyperparasitised by *A. alternatum*.

Vidal and colleagues did pioneer work on the effect of *A. alternatum* on herbivory and plant compounds associated with it (Raps and Vidal 1998). They measured carbon-nitrogen ratios in leaves of unolonised and colonised plants and did not find a difference. Li (2008) on the other hand detected an elevated phytosterol content in rapeseed leaves after inoculation with *A. alternatum*.

In the group of Ludwig-Müller the effect of the endophyte on clubroot infected plants was studied (Jäschke et al. 2010). After co-inoculation with *A. alternatum* a reduction of clubroot symptoms, i. e. smaller galls, were found in Arabidopsis. In Chinese cabbage a reduction of symptoms by 40% was achieved (Doan et al. 2010). Also shoot biomass was increased upon inoculation with *A. alternatum* (Doan et al. 2008). Four weeks after

inoculation Chinese cabbage galls with the endophyte showed less cells with resting spores of the pathogen, indicating a time lag in spore formation of the pathogen.

All papers report observed effects rather than a study of the mechanism behind the control potential of this fungus. Therefore the main goal of this thesis was to study the molecular basis of the observed biocontrol effects against the clubroot pathogen.

Table 1.1.: References that mention *A. alternatum* in the context of biocontrol.

Reference	Plant host	Antagonist	Observed effects	Mechanism
Malathrakis (1985)	cucurbits	powdery mildew *Sphaerotheca fuliginea*	hyper-parasitism	parasitism
Bettiol (1996)	coconut	tar spot *Coccostroma palmicola, Catacauma torrendiella*	reduced infection	mycoparasite
Romero et al. (2003)	melon	powdery mildew *Podosphaera fusca*	reduced infection	mycoparasite
Kasselaki et al. (2006)	tomato	powdery mildew *Leveillula taurica*	reduced infection	presumably induction of resistance
Jäschke et al. (2010)	thale cress	clubroot *Plasmodiophora brassicae*	reduced symptoms	presumably induction of resistance
Doan et al. (2010)	thale cress, Chinese cabbage	clubroot *Plasmodiophora brassicae*	reduced symptoms	presumably induction of resistance

1.4. The plant immune system

Plants have developed several resistance mechanisms in order to prevent successful overtake by pathogens and herbivores. It is estimated that over 80% of the land plants are colonised by microorganisms (Van Der Heijden et al. 2008), with the plant root being the main colonised organ. An enormous amount of bacteria and fungi live within land plants - average densities are estimated to range from 10^4 to 10^7 cfu per gram fresh weight (Backman and Sikora 2008).

Plants benefit from microbes that live inside the root and constitute the root microbiome in various ways: microbes provide enhanced mineral and nutrient uptake, fix ni-

trogen, promote growth and protect the plant from invaders. The ability of microbes to protect their host plant was observed in many studies but the mechanism behind it is not well understood (Cook et al. 1996; Niu et al. 2011; Pieterse et al. 2014).

It is clear that the mutualistic relationship between microbes and plants is based on an equilibrium that requires a constant molecular dialogue. Upon recognition of an invader, plants respond with an accumulation of signalling molecules such as plant hormones. The activation of defence mechanisms is usually not restricted to the site of the infection but also effective in tissues that are spatially separated from the tissue where the infection takes place, a process called "induced resistance" (Pieterse et al. 2014).

The term "systemic acquired resistance" (SAR) was coined in the 1960s and refers to the phenomenon in which uninfected plant parts become more resistant in response to a localized infection elsewhere (Ross 1961). SAR requires the presence of salicylic acid (SA) that accumulates in systemic tissues upon infection by biotroph pathogens (van Wees et al. 2000). Markers for SAR in Arabidopsis are the *PATHOGENESIS-RELATED PROTEIN-1* gene (*PR1*) and the *NONEXPRESSOR OF PR GENES-1* (*NPR1*) (Ryals et al. 1996).

In 1991 three independent research groups discovered that nonpathogenic microbes from the rhizosphere can induce systemic resistance, shortly ISR (Alström 1991; Van Peer et al. 1991; Wei et al. 1991). ISR develops without the accumulation of pathogenesis related (PR) proteins and is independent of SA. In the last years several studies proved that it is common for beneficial microbes to activate the SA-independent ISR pathway. The accumulation of jasmonic acid (JA) and/or ethylene (ET) is distinctive for ISR and leads to the activation of JA- and ET-responsive genes (Van Loon et al. 1998; Van Wees et al. 2008). Marker genes for ISR are the *PLANT-DEFENSIN-1.2* (*PDF1.2*) and *Thionin (THI2.1*), (Zhang et al. 2007, Onate-Sanchez et al. 2007). According to reviews on that topic, priming is the basis of ISR (Heil and Bostock 2002; Van Wees et al. 2008; Pieterse et al. 2014; see next section).

Besides JA and SA other plant hormones participate in the immune response of the plant as well and can shape the outcome and effectiveness of the defence response. To prioritise the growth of plants, auxin, cytokinin and gibberellins can repress defence pathways and vice versa; to push defence mechanisms SA and JA can suppress the action of these hormones and thus act on the expense of plant growth. In general, the plant hormones ET and abscisic acid (ABA) act synergistically with JA-regulated defence mechanisms and antagonise the SA response (Pieterse et al. 2012).

1.4.1. Transcriptional regulation of defence responses

Both SA- and JA/ET-controlled signaling pathways are primarily regulated at the level of gene transcription.

Upon recognition of the pathogen the genes *SID2* and *GH3.5* are activated and lead to

the synthesis and accumulation of SA and induce the migration of NPR1 from the cytosol to the nucleus (Zhang et al. 2007; Shah 2010; Fig. 1.2). Downstream of SA the redox-regulated protein NPR1 controls SAR signaling by acting as transcriptional coactivator of numerous *PR* genes (Pieterse and Van Loon (2004)). NPR1 interacts with transcription factors of the TGA family that bind together with WRKY transcription factors to the promotors of SA-responsive defence genes and activate them (Pieterse et al. 2014). The other branch of the plant defence response is dependent on JA and ET: JAR1 catalyzes the formation of JA-Ile which in turn activates the SCF-COI1 receptor complex. This complex activates the transcription of *ETR1*, *PDF1.2* and *THI2.1* (Pieterse et al. 2012). *COI1* is the key component in the JA-responsive pathway, regulating the expression of other JA-responsive genes. Low levels of *COI1* indicate a deactivated JA-pathway and lead to a loss of resistance against TMV (Liu et al. 2004).

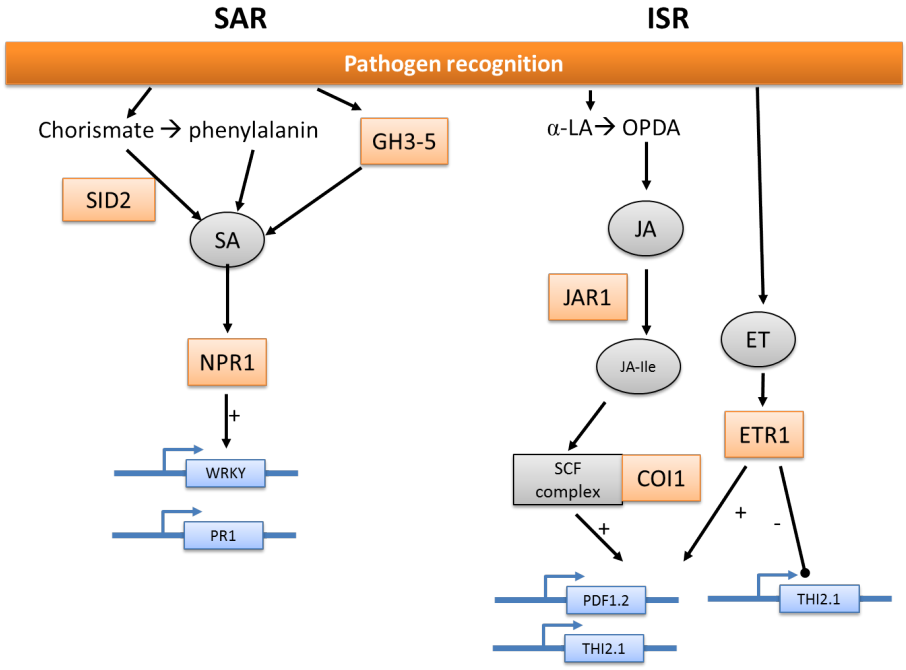


Figure 1.2.: Interplay of the SA- and JA/ET-mediated resistance pathways. Orange and blue boxes indicate genes investigated in this study. Abbreviations: SA - salicylic acid, JA - jasmonate, ET - ethylene, SAR - systemic acquired resistance, ISR - induced systemic resistance. Modified after Turner et al. 2002; Devoto and Turner 2003; Eulgem 2005; Zhang et al. 2007; Pieterse et al. 2012.

1.4.2. Pathogen recognition and priming

To address the attack of phytopathogens, plants have developed a complex feedback system that involves at least 2 lines of defence and is dependent on the amplitude of the signals induced by the pathogen. In 2006 Jones and Dangl published their highly cited zigzag model which describes how the plant perceives pathogen-associated molecular patterns (PAMPs) and what follows after the detection of these signals (Fig. 1.3). The first line of defence - called PAMP-triggered immunity or shortly PTI - is effective against

most invaders and hinders further colonisation by the pathogen (Jones and Dangl 2006; Pieterse et al. 2014). Successful pathogens either suppress PTI or prevent detection by the host. If this occurs, the second line of defence comes into action: the effector triggered immunity or ETI. This defence line is mediated over R proteins that belong to the NB-LRR (nucleotide-binding-leucine-rich repeat) receptor proteins. In the theory of Jones and Dangl (2006) a successful pathogen constantly develops new effectors that suppress the PAMP-triggered immunity and thus establishes itself in the plant, causing visible symptoms.

In detail, the model of Jones and Dangl (2006) is constituted by 4 phases. In phase 1, PAMPs or MAMPs are detected by pattern recognition receptors (PRRs) and lead to PTI that can hinder further colonisation with the pathogen. A typical elicitor of PTI is flagellin which is recognized by the flagellin receptor FLS2. In phase 2, successful pathogens suppress PTI by expressing effector molecules thus causing effector-triggered susceptibility (ETS). In phase 3, NB-LRR proteins recognise 1 or more effectors and activate effector-triggered immunity (ETI) which is an amplified version of PTI. In phase 4, pathogens develop new effectors to suppress ETI which in turn lead to new NB-LRR alleles to detect these new effectors and consequently lead to ETI again (Jones and Dangl 2006). To date, still many details of this complex system are not well understood.

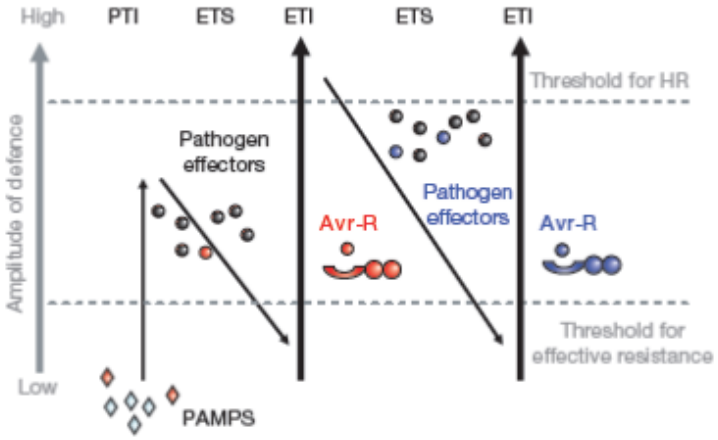


Figure 1.3.: The highly cited zigzag model of Jones and Dangl (2006) illustrates the quantitative output of the plant immune system. Briefly described, PAMPs are recognized by the plant and result in the low amplitude-PTI response. Successful pathogens deliver effectors (red dot) that cause ETS and result in the higher amplitudal ETI. Evolution favours the development of new pathogen effectors to overcome ETI and the plant in turn develops new disease resistance proteins (Avr-R). Abbreviations: PAMP - pathogen associated molecular pattern, PTI - pathogen-triggered immunity, ETS - effector-triggered susceptibilty, ETI - effector-triggered immunity, Avr-R - disease resistance proteins, HR - hypersensitive cell death response.

However, the involvement of several genes in this immune system is relatively sure. Microbe-associated molecular pattern (MAMP)-responsive genes such as *MAP* and *FLS2* are involved in the deposition of callose and the BRI1 ASSOCIATED RECEPTOR KINASE

BAK1 activates *PR1* and *PDF1.2*.

The detection of PAMPs/MAMPs (both terms are synonym) leads to priming of the plant. Priming is a cost-effective way for the plant to activate inducible defence-responses upon pathogen attack. After perceiving a priming-relevant inducer the plant prepares to activate inducible defence mechanisms and the state of enhanced alertness leads to a faster and stronger activation of defence mechanisms in comparison to non-primed controls (Vos et al. 2013). Priming results in the potentiated expression of SA- and JA/ET-inducible resistance genes.

A scheme of the involvement and interaction of MAMP-responsive genes from this study can be found in Fig. 1.4.

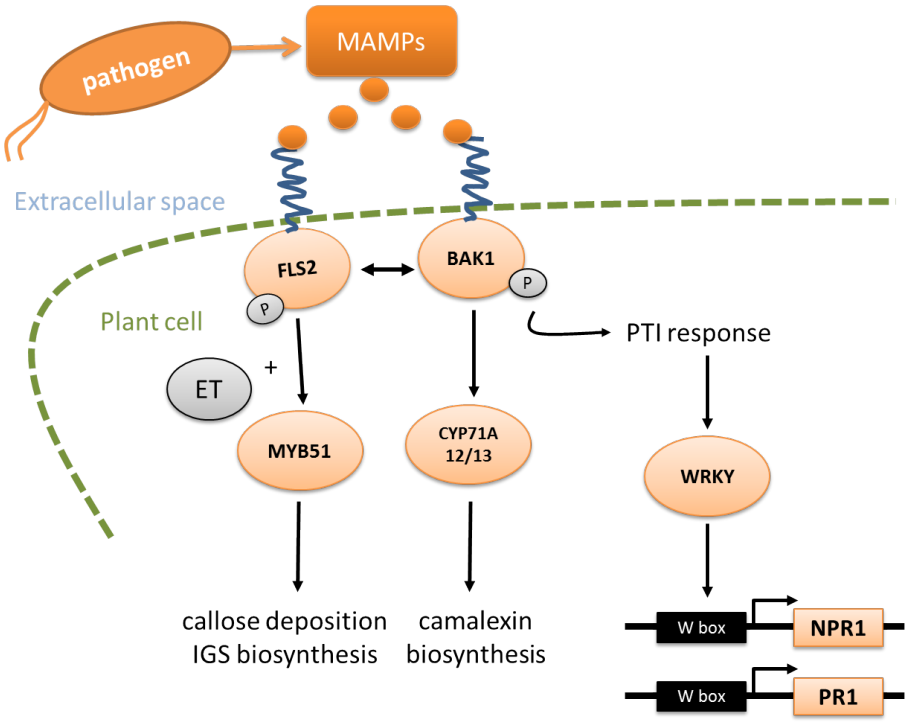


Figure 1.4.: Modell of pathogen recognition with MAMP-responsive genes investigated in this study. This modell combines findings from several literature sources (Singh et al. 2002; Xu et al. 2008; Clay et al. 2009; Dodds and Rathjen 2010). Abbreviations: MAMP - microbe-associated molecular pattern, PTI - pathogen-triggered immunity.

1.5. Aim and relevance of this project

The results of 2 earlier studies (see 1.3.1) suggest that an induction of resistance is the cause for the biocontrol effects observed. By switching on the "defence mode" plants can minimise at least partly the negative effects that follow a pathogen infection.

Induced resistance can be assessed molecularly by quantifiying transcript levels of defence related genes and microscopically by looking at defence structures from the plant such as callose depositions in affected tissues.

Possibly the endophyte *A. alternatum* produces toxic compounds that negatively affect the growth of *P. brassicae* in the plants. A sister clade of *A. alternatum*, *A. zeae* is known to produce toxins that have antifungal potential (Pohling et al. 2008). So far *A. alternatum* is not known to produce any toxins. A germination assay with *P. brassicae* spores did not show a negative effect of the endophyte on germination of resting spores of the pathogen (Jäschke et al. 2010).

It has been repeatedly reported in the last years that the clubroot disease spreads throughout Germany (J. Ludwig-Müller, TU Dresden; E. Diederichsen, FU Berlin; Sächsisches Ministerium für Umwelt und Landwirtschaft [SMUL], pers. comm.; Diederichsen et al. 2014). Since no sustainable and lasting control is available for this disease (see 1.1.3) the urgent need arises to find mechanisms to circumvent large outbursts of the pathogen as experienced in Australia 30 years ago (see section 1.1.3). Since 2014 farmers in Saxony are advised to use resistant cultivars on fields were clubroot has been reported (Sortenempfehlungen für Winterraps 2014, SMUL). It is expected that the disease will spread in the following years with consequences that cannot be foreseen today.

The aim of this study is to contribute to the understanding of the molecular mechanisms of pathogen-plant interactions in general and the very specialised interaction of *Plasmodiophora* with Arabidopsis in particular.

The focus of this work is on the molecular mechanisms behind the interaction of *P. brassicaea* and *A. alternatum* with Arabidopsis. Alternative methods to control plant diseases are urgently needed but the basis of their application often lacks fundamental knowledge. By the use of qRT-PCR, changes in the transcript levels of genes from Arabidopsis root tissue upon pathogen challenge will be detected. The point of interaction of both pathogens within the host plant will be identified by histological analyses. Furthermore the wider potential of *A. alternatum* as biocontrol agent will be studied with *in vitro* tests.

1.6. Objectives

In general the objectives of this study are to:

i) analyse the activity of Arabidopsis genes involved in resistance pathways in roots in response to *A. alternatum* and *P. brassicae* infections,

ii) quantify the impact of *P. brassicae* on rapeseed development in 2 German rapeseed cultivars,

iii) test inactivated spore solutions of the endophyte against the clubroot disease in Arabidopsis and rapeseed,

ii) assess the biocontrol potential of the root endophyte *A. alternatum.*

These goals will be achieved by carrying out the following work programme:

(1) Interaction of P. brassicae and A. alternatum with the model plant A. thaliana

Arabidopsis plants will be inoculated with a spore suspension of *P. brassicae* and *A. alternatum*. For this the plants will be divided in 4 groups; 1 control group treated with buffer; 1 group treated with *A. alternatum*, 1 group treated with *P. brassicae* and 1 treated with both organisms. The roots of plants will then be analysed by means of a disease rating, microscopy and by assessing the overall developmental stage of the plant. If applicable, biomass and stem lengths will be recorded.

(2) Identification of resistance genes in the tripartite interaction between Plasmodiophora, Acremonium and Arabidopsis

From root tissue of inoculated and non-inoculated Arabidopsis plants mRNA will be extracted, followed by cDNA synthesis and analysis with (s)qRT-PCR to detect changes in the transcript levels of putative resistance genes in this complex interaction. Findings on the gene expression level will be confirmed by the analysis of mutants and overexpressor lines of the respective genes.

Additionally, a microarray on Arabidopsis roots will be carried out to depict the events at the stage of initial root hair infection.

(3) Verification of results in B. napus

Inoculation experiments as described above and subsequent disease rating and microscopy of root tissue will be performed on 2 German varieties of rapeseed. In addition, mRNA root tissue will be analysed by qRT-PCR using primer pairs for putative resistance genes that are homologue to those from Arabidopsis. The amount of fungal and protist DNA from different plant tissues will be determined with qPCR.

(4) Biocontrol potential of A. alternatum and economical relevance

A. alternatum is a relatively unknown fungus so far. Therefore, the enzymatic activity of the endophyte and its competitive behaviour against other phytopathogenic fungi will be studied. For this a fungus-versus-fungus assay will be carried out on agar plates and the growth of both species will be recorded. Additionally, enzymatic activities will be determined *in vitro*.

To assess the role of plant hormones in the interaction of *A. alternatum* with Arabidopsis, hormone mutants will be grown on agar plates and inoculated directly and indirectly with the fungus. To test if the endophyte increases the salt stress tolerance of Arabidopsis, seedlings will be grown on agar plates with low concentrations of NaCl and mannitol and inoculated with *A. alternatum*.

For a potential application of this fungus in integrative pest management in the future, solutions containing inactivated spores of *A. alternatum* will be tested on Arabidopsis and *B. napus* and the symptom development will be recorded using the parameters specified

in (1).

2. Materials and Methods

2.1. Overview of all plant experiments

According to the work schedule outlined in section 1.6, a series of inoculation experiments was carried out in order to achieve the goals set for this project. Several parameters (spore density, substrate type, harvest points) were varied according to the specific goal of each experiment. To obtain a better overview of the successful experiments used for molecular studies see table 2.1.

Table 2.1.: Overview of successful inoculation experiments with *A. alternatum* and *P. brassicae* used for molecular studies. The setup of experiments is described in section 2.3.2.1. RNA was extracted from the roots and transcribed to cDNA, followed by RT-PCR. No experiments with *P. brassicae* could be carried out on agar plates as this protist is not compatible with axenic cultures. Abbreviations: *Aa - A. alternatum*, *Pb - P. brassicae*, dai - days after inoculation.

plant species	substrate	inoculation	harvest (dai)	RT-PCR	trials
A. thaliana Col-0	soil	*Aa, Pb*	9 14 20	semi-quantitative	2
A. thaliana Col-0	sand	*Aa, Pb*	9 14 20	quantitative (real time)	2
B. napus var. Visby	sand	*Aa, Pb*	3	quantitative (real time)	2
A. thaliana Col-0	agar; HL medium	*Aa*	1 3 4	quantitative (real time)	1

For the disease quantification plants were grown on soil and rated as described in 2.3.3.2. The interaction of *A. alternatum* with Arabidopsis wildtype and mutants was monitored on agar plates. Samples for microscopy were taken after the experiments were finished.

2.2. Materials

2.2.1. Plants

Arabidopsis thaliana ecotype Columbia (Col-0) was used in all experiments with Arabidopsis, except when stated otherwise. A complete overview of all utilised plants from this study is given in Table 2.2 and 2.3.

For *Brassica napus* local seeds that are used by farmers in Saxony were chosen. Seeds of the winter rapeseed variety "Visby" were obtained from Dr. Wolfgang Karalus in 2012 (Sächsisches Ministerium für Umwelt und Landwirtschaft, Nossen), the summer variety "Ability" was obtained in the same year from the Deutsche Saatgutveredlung, Lippstedt. *B. napus* is a cross of *B. oleracea* (n=9, CC-genome) and *B. rapa* (n=10, AA-genome) thus having a tetraploid genome AACC (n=19) and displaying properties of both species.

Table 2.2.: Wildtype plants used for inoculation tests. Abbreviations: NASC - The European Arabidopsis Stock Centre, SMUL - Sächsisches Ministerium für Umwelt und Landwirtschaft, DSV - Deutsche Saatgutveredlung.

Species	Genotype	Abbreviation	Origin
Arabidopsis thaliana	Columbia	Col-0	NASC
	Enkheim	En-2	NASC
	Landsberg erecta	La er	NASC
Brassica napus	subsp. oleifera var. Visby		SMUL
	subsp. oleifera var. Ability		DSV

Table 2.3.: Arabidopsis mutants and overexpressor lines used for inoculation tests. Abbrevations: NASC - The European Arabidopsis Stock Centre, ABRC - Arabidopsis Biological Resource Center.

Type	Characteristics	Affected hormone	Background	Source
tir1	deficient auxin receptor TIR1	auxin	Col-0	M. Estelle, USA
afb1-3	deficient auxin receptor AFB1-3	"	"	"
afb1-2,2-3	deficient auxin receptor AFB1-2,2-3	"	"	"
cyr1	cytokinin insensitive	cytokinin	"	ABRC
ckx2	deficient in cytokinin oxidase 2 signaling	"	"	NASC
jar1	jasmonate insensitive	jasmonate	"	ABRC
eto2	ethylene overproducer	ethylene	"	"
eto3	ethylene overproducer	"	"	"
bri1-6	deficient brassinosteroid receptor	brassinosteroid	En-2	NASC
aba1	ABA insensit-ive/deficient	abscisic acid	La er	A. Kranz, Frankfurt
aux-1	auxin insensitive	auxin	"	ABRC
wrky18	knock-down of *WRKY18*	-	Col-0	U. Zentgraf, Tübingen
w18Oe	overexpression of *WRKY18*	-	"	U. Zentgraf, Tübingen

2.2.2. The endophytic fungus A. alternatum

The fungus *Acremonium alternatum* strain CBS 831.97 used in this study was obtained from the laboratory of Stefan Vidal, Göttingen and verified microscopically and by PCR for the correct classification. Primers specifically designed for *A. alternatum* were obtained from Petr Karlovskys laboratory, Göttingen (Jäschke et al. 2010, Table 2.10). These

primers also target *A. strictum*. However, *A. strictum* has a different sporulation procedure and does not grow inside of leaves or stems but remains in the root. Throughout all experiments with *A. alternatum* plant material was routinely tested with PCR for the presence of the fungus.

2.2.3. The clubroot pathogen P. brassicae

The single spore isolate ´e3´ was used in all experiments. This isolate was characterised by Fähling et al. (2003) and Graf et al. (2004).

2.2.4. Growth media

Plants were mainly cultivated on Hoagland medium which ensures adequate root growth. The endophyte *A. alternatum* was routinely cultured on PDA, in experiments where the phosphate solubilising potential of the fungus was examined. The medium from Sundara and Sinha (Posada et al. 2013) proved to be superior to PDA for cultivation of *A. alternatum*. A complete list of all standard media can be found in table 2.4.

Table 2.4.: Standard growth media for plant and fungus cultivation.

Media		Preparation per 1 liter	pH	organism	Source
Hoaglands Medium	HL	1,6 g Hoaglands salt no. 2, 0,5 g MES, 10 g sucrose, 10 g agar	5,6	plant	Sigma
Murashige-Skoog-Medium	MS	4,4 g MS salt, 10 g sucrose, 10 g agar	5,8	plant	Sigma
Potato dextrose agar	PDA	26,5 g PDA, 10 g agar	5,6	fungus	Roth
Sundara and Sinha medium	SS	0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.3 g $MgSO_4 \times 7 \times H_2O$, 0.004 g $MnSO_4 \times H_2O$, 0.002 g $FeSO_4 \times 7H_2O$,0.2 g NaCl, 10 g D-glucose, 0.5 g yeast extract, 18 g agar	6,8	fungus	Posada et al. 2013

To test for enzymatic activities the fungus was grown on indicator media described from the literature (see Table 2.5).

Table 2.5.: Growth media for enzymatic tests.

Media	Enzymatic activity	Preparation per 1 liter	pH	Source
Skimmed milk agar (MA)	protease	500 ml sterilized milk are mixed with 500 ml 4% water agar at 55°C	-	Zheng et al. (2011)
Carboxymethyl-cellulose-agar (CMC)	cellulase	10 g peptone, 10 g yeast extract, 10 g carboxymethylcellulose, 5 g NaCl, 1 g KH_2PO_4, 18 g agar	7.0	"
Chitin-agar (CA)	chitinase	1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4$, 1% (w/v) colloid chitin, 20 g agar	7.0	"
Nitrogen fixing bacteria medium (NFb)	nitrogen fixation	5.0 g malic acid, 0.5g K_2HPO_4, 0.2 g $MgSO_4$*$7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2$, 0.5 % bromothymol blue in 2 ml 0.2 N KOH, 1 ml vitamin solution containing 100 mg/l biotin and 200 mg/l pyridoxine-HCl (vit. B6), 2 ml micronutrient solution (0.4 g/l $CuSO_4$, 0.12 g/l $ZnSO_4$4×$7H_2$2O, 1.4 g/l H_2BO_3, 1 g/l Na_2MoOx×$2H_2O$, 1.5 g/l $MnSO_4 \times H_2O$), 4 ml 1.64% Fe-EDTA solution, 4.5 g KOH, 18 g agar	6.8	"
Calcium phosphate medium (PCa)	phosphate solubilisation	see SS medium, additionally phosphate solution with 0.5 g Arabic gum, 0.5 g $Ca_3(PO_4)_2$	6.8	Posada et al. (2013)
Potassium phosphate medium (PFe)	phosphate solubilisation	see SS medium, additionally phosphate solution with 0.5 g Arabic gum, 0.5 g $FePO_4 \times H_2O$	6.8	"

2.2.5. Molecular and chemical supplies, technical equipment

Molecular reagents were obtained from ThermoScientific, Roth and VWR. Chemicals, including staining solutions, were obtained from Roth and Sigma and are described in the text together with the respective method. Technical equipment for standard applications in molecular biology such as thermomixers and power supplies for gel electrophoresis are not further described. A list of special technical equipment used in this study can be found in table 2.6.

Table 2.6.: Special technical equipment used in this study.

Description	Purpose	Manufacturer
Bioanalyzer	Instrument for the measurement of RNA integrity	BioRad
nanodrop ND-1000	spectrophotometer for measuring nucleic acid concentrations	PeqLab
qTower 2.2	PCR cycler for real-time PCR	Jenaanalytik
ZEISS Supra 40VP	Scanning electron microscope	Zeiss

2.3. Methods

2.3.1. Plant cultivation and growing conditions

2.3.1.1. Development of a hydroponic culture system

The roots were the major organ studied and analysed in this thesis. Roots comprise only a small part of the plant respective to the upper plant organs. This is strongly reflected in the biomass and requires therefore a large number of plants to be analysed with each treatment condition to gain enough material for representative RNA analyses and disease ratings.

The major part of this work was done with Arabidopsis. This model plant is relatively simple to cultivate but has a small biomass. Therefore, and for statistical reasons, the aim was to study at least 20 or 30 plants per treatment in each experiment. All experiments were repeated at least once.

Three types of plant cultivation were used in this study. It has been observed that *P. brassicae* infections are best validated on soil since the galls achieve their largest state in a soil environment whereas on sand or other substrates gall development can be impaired (J. Ludwig-Müller, pers. communication). Hence plants used for disease ratings of the roots were grown on soil as long term experiments in greenhouses or climate chambers.

The careful harvest of roots out of soil for RNA extraction takes a long time and subsequent analysis can suffer from this time lag.

For experiments that involved several harvests during the growing period, i. e. the gene expression studies on roots, a hydroponic culture system was developed, validated and established. This method met the following criteria: high number of plants per harvest and treatment ($\geq$30) for good statistics, preferably quick (circadian gene regulation) and non-invasive (RNA degradation) harvest procedure for roots (to determine root fresh weight) and a relatively simple handling. This simple and easy to use hydroponic culture

system was based on 2 papers on that topic (Donald et al. 2004; Smeets et al. 2008). The setting is described below (2.3.1.3, see also Fig. 2.1).

The third type was the cultivation on agar plates with MS or Hoagland medium. Here the harvest of roots can be done within seconds and a large number of plants can be analysed at once.

Details of the cultivation are described in the sections 2.3.1.2 to 2.3.1.4.

2.3.1.2. Plant cultivation on soil

For cultivation on soil Arabidopsis seeds were stratified at 8°C in tap water for 48 to 72 hours in the dark prior to sowing. *Brassica* seeds were washed free from their insectizide cointaining-coating and pre-germinated on moist filter paper in glass petri dishes in the light for 2 or 3 days. Standard soil type "Pikiererde Classic Profisubstrat" (Einheitserde) was sieved coarsely and then steam sterilized for 120 min. After cooling down the soil was mixed with sand („Kinderspielsand" baked at 60°C for at least 1 day) in the relation soil:sand 3-4:1 and adequately watered prior to sowing. For Arabidopsis cultivation the soil mixture was spread in trays (17x24x5 cm) and 20 to 48 seeds were sown per tray and watered from below. A lid was placed on the trays for the first 2 weeks of the gowth period to ensure adequate moisture for the seedlings.

Emerging Brassica seedlings were planted in 11x11x12 cm pots (2 seedlings/pot) filled with the soil mixture and put in a tray with water.

Plants grew in climate chambers under long day conditions (16 hours light at 23 ± 1°C, 8 hours dark at 18 ± 1°C) (Arabidopsis) or in the green house with a temperature range from 14 to 27 °C and a relative humidity of 40 to 80 % (*Brassica*).

The *WRKY18* overexpressor lines used in this study were not homozygous. Therefore *wrky18oe* seeds of 2 lines were mixed with sand, spread on the earth surface and sprayed with 0,1 % BASTA after germination for 2 times for the selection of transgenic plants. Surviving plants were then individualised to seperate trays with the soil:sand mixture and treated with spore suspensions as described below.

2.3.1.3. Plant cultivation on sand (hydroponic culture)

For hydroponic cultivation Arabidopsis and *Brassica* seeds were surface sterilized in a sodiumhypochlorit:water-mixture 3:2 for 5 to 10 minutes. Seeds were washed with sterile water 6 times after sterilizing. Arabidopsis seeds were stratified as mentioned above while Brassica seeds were pre-germinated. The hydroculture system was set up as follows: 10 ml pipette tips containing a mixture of coarse and fine baked sand („Kinder-spielsand" baked at 60°C for at least 48 hours) were watered with autoclaved, distilled water until thoroughly wet and placed in a rack. Seeds were sown on the sand surface of the pipette tips so that each tube contained 3 seeds. The rack containing 93 pipette

tips was then put in a plastic box with lid using one box per treatment and placed in the greenhouse (*Brassica*) or climate chamber (Arabidopsis; see 2.1). Because germination rate is often a problem on sand, additional pipette tips with seeds were prepared and placed in the climate chamber. Relative humidity was 80-90% and the temperature was 19-23°C/23°C (greenhouse/climate chamber) throughout the growing period. After germination (5 days after sowing) plantlets were thinned to 1 plant per pipette tip. Every 1 or 2 days each individual plant was watered with approximately 100 µl nutrient solution (Smeets et al. 2008) pipetted carefully on the sand next to the plant.

Figure 2.1.: Setup of the custom-made hydroculture system for Arabidopsis and *Brassica* cultivation. Left: each tray consists of a plastic plate with 4 legs and has a capacity of 93×5 ml pipette tips; right: close-up of hydroponically grown Arabidopsis, 30 days old.

2.3.1.4. Plant cultivation on agar plates

Plant seeds were surface sterilised and stratified as described in 2.3.1.3. The inoculation took place 2 weeks after sowing. A spore suspension of *A. alternatum* was diluted with autoclaved distilled water to a concentration of 10^6 to 10^7 spores/ml. 200 µl of this suspension was then pipetted directly on the roots, usually some mm below the seed. Treated plates remained under the sterile flow until the water had dried off and were then sealed with parafilm and put in the climate chamber at long day conditions.

For the indirect interaction Arabidopsis plants were grown as described above. Seven days after sowing a spore suspension of *A. alternatum* (concentration 8x10^7) was applied at the bottom of the plate to prevent direct contact of roots and fungus. These plants were harvested 7 days after treatment.

2.3.1.5. Determining the growth stage of plants

One aim of this study was to find out how the endophytic fungus influences the overall growth of plants. During the first experiments it became obvious that Arabidopsis

infected with *A. alternatum* flowered earlier and had more flowers in total. To address this shift in senescence for all following experiments the growth stage of plants was recorded according to Boyes et al. (2001). The key growth stages used were principal growth stage 3 (rosette development until completion), 5.1 (first flower buds visible) and 6.0 - 6.9 (flower production). For a better overview the 2 phases vegetative growth (leaf development) and generative growth (stem elongation and flower development) were chosen. The key was mainly applied to plants in plate experiments. Where appropriate the number of flowers was recorded.

The developmental stage of the crop plant *Brassica napus* was rated according to the BBCH-scale of farmers (Meier 2001). Here the stages 1 (leaf development), 3 (stem elongation), 5 (inflorescence emergence), 6 (flowering) and 7 (development of fruit) were used.

2.3.2. Pathogen inoculation and detection

2.3.2.1. Inoculation of plants with spore suspensions

For experiments on soil Arabidopsis plants were inoculated 14 days after sowing with 2 ml spore suspension per plant. Rapeseed plants were inoculated 5 days after sowing with 3 ml inoculum per plant. For hydroponic culture experiments 200 µl inoculum were used for both plant species by carefully pipetting of the spore suspension on the substrate next to the plant. There were 4 groups: 1 control group treated with 50 mM KH_2PO_4 (pH 5.5); 1 group treated with a spore suspension of *A. alternatum*; 1 group treated with a spore suspension of *P. brassicae* and 1 group treated with a spore suspension containing spores of both organisms. The usual spore concentration used was 10^7 spores/ml. With this concentration a disease index of roughly 100% was achieved in most of the experiments. Also milder concentrations of 10^5 and 10^6 were used, mainly to test for susceptibility of Arabidopsis mutants. All spore suspensions were adjusted to the desired concentration using the same 50 mM KH_2PO_4 buffer that was used for the control group.

2.3.2.2. Inoculation of plants with cell wall extract and autoclaved spores of A. alternatum

To test whether parts of the fungus are sufficient to facilitate resistance against the club-root pathogen, Arabidopsis and rapeseed plants were inoculated with a suspension of autoclaved spores and a crude cell wall extract (CWE; see section 2.3.5.2).

2 experiments were carried out with Arabidopsis. In the first experiment the cell wall extract and autoclaved spores (=priming solutions) were administererd at the same time as the *P. brassicae* inoculum 14 days after sowing. Two ml of each solution was used. In a second experiment the plants were pre-treated with the priming solutions 4 days before inoculation with *P. brassicae*. Here 4 ml of CWE (= 4%) and 2 ml of autoclaved

spores (density 2x10^6 spore/ml) was used. The disease rating was done 4 weeks after treatment.

With rapeseed 1 experiment was carried out with the priming solutions. The CWE was adjusted to a pH of 5,7 with KH_2PO_4. Three days after sowing *B. napus* seedlings (variety "Ability") were inoculated with 2 or 4 ml of a 1% CWE solution and 3 ml of autoclaved spores (2x10^6/ml) of *A. alternatum*. Four days later the plants were inoculated with *P. brassicae* (2x10^6 spores/ml). The disease rating for *B. napus* was done 6 to 9 weeks after inoculation with *P. brassicae*.

2.3.3. Sample preparation: harvesting, growth status, disease evaluation

2.3.3.1. Harvesting procedure for roots

For the disease rating plants were gently removed from the soil and the roots were washed free of remaining soil, using a brush for support.

For all experiments that involved RNA extraction a working procedure was established that ensured maximum quantity of root fresh weight at a minimal time effort to prevent a bias in the experiments due to the circadian regulation of most genes. Hydroponically cultured plants were harvested by putting the pipette tips in a tray with water and carefully pulling at the plant until it came off. Excess sand was gently removed by using a brush. Whole plants remained in the water until every plant from every treatment group was harvested. Roots were then cut off at the same time point for each treatment group, weighed and immediately processed for RNA extraction with RNAzol. Treatments were always harvested in the same order, starting with the controls and followed by *A. alternatum* inoculated, mixed inoculation and *P. brassicae* inoculated. By this procedure the time frame for harvest was reduced to 1 or 2 hours for each harvest.

2.3.3.2. Disease rating

Clubroot symptoms in Arabidopsis were assessed by rating the roots of infected plants after a scheme of Klewer et al. 2001 (see table 2.7). A disease index (DI) according to Siemens et al. 2002 was calculated that describes the severity of the infection of one experiment. The DI is calculated after the formula

$$DI = \frac{(1n+2n_2+3n_3+4n_4)\times 100}{4N_{total}}$$

where $n_1$to n_4 is the number of plants in the indicated disease class and N_{total} is the total number of plants tested.

For *Brassica* the scale of Yoshikawa et al. (1977) was used (Table 2.7; Fig. 2.2).

Table 2.7.: Disease rating classes for Arabidopsis and *Brassica* after Klewer et al. (2001) and Yoshikawa et al. (1977).

Disease class	Symptoms in Arabidopsis	Symptoms in *Brassica*
0	no symptoms	no symptoms
1	very small clubs on lateral roots	very light swelling on lateral roots
2	small clubs covering main root and few lateral roots	moderate swelling on lateral/main roots
3	medium to bigger sized clubs including main roots, plant growth can be impaired	severe swelling on lateral/main roots
4	severe clubs in lateral, main root or rosette, fine roots destroyed, plant roots impaired	severe swelling or decay on lateral/main roots

Measuring the size or biomass of galls is not applicable as a measure of the extent of the disease (Rausch et al. 1981; Siemens et al. 2002). On the one hand the relative size of a gall is positively correlated with the overall health of the plant, i. e. generally smaller plants tend to have smaller galls in comparison. On the other hand gall size can be reduced due to the presence of resistance alleles (Rausch et al. 1981). Additionally, during harvesting the galls out of soil remaining roots often get detached from the gall and are lost for determination. Therefore in most experiments the fresh weight of above ground plant parts of disease rated plants was determined but not the root weight.

The results from the disease rating were analysed statistically with the Kruskal-Wallis test, a non-parametric one-way analysis of variance by ranks. The test was carried out with the software SPSS (version 22).

There is still an ongoing discussion what is regarded as resistant and what is moderately resistant/tolerant in the clubroot interaction with its host plants.

To define if the plants were susceptible or resistant to the clubroot pathogen after treatment with *A. alternatum*, cut-off values were defined (J. Ludwig-Müller, E. Diedrichsen, pers. communication). Plants with a DI of $\geq$90 were termed as susceptible, a DI betwen 40 and 70 moderately resistant/tolerant and a DI of $\leq$40 resistant.

2.3.4. Propagation of P. brassicae spores

To obtain a spore suspension of the obligate biotroph clubroot pathogen 1 week-old Chinese cabbage plants were inoculated with a spore solution of *P. brassicae* (10^7 spores/ml). Mature galls were then harvested and frozen in liquid nitrogen. The galls were homogenised with a blender and the homogenate was filtered through gauze. After centrifugation (2 times, 2500 g, 10 min) the resting spores were collected from the bottom of the tube and resuspended with destilled water. The spore concentration was determined

Figure 2.2.: Disease classes in rapeseed roots after inoculation with *Plasmodiophora brassicae*. Numbers indicate the disease class according to Yoshikawa et al. (1977) (see Table 2.7).

under the microscope with a Neubauer counting chamber.

2.3.5. Fungal cultivation and preparation of priming solutions

2.3.5.1. The fungus A. alternatum, spore suspension preparation, viability test

To obtain a spore suspension for the inoculation experiments pieces of agar containing the fungus were placed in Erlenmeyer flasks with PDA brooth and kept in a rotary shaker for 2 to 3 weeks at 24° to 28°C. The growth of the fungus was checked routintely by microscopy. The spores were obtained by filtering the medium containing the fungus through cheese cloth. Spore density was then determined using Neubauers improved counting chamber and a hemocytometer. Spore suspensions were kept frozen in sterile Eppendorf tubes and stored at - 20°C until used.

A viability test was performed by inoculating tomato plants with the fungus and reisolating it out of leaves. For this, tomato leaves of inoculated plants were surface sterilized with ethanol (70%) and placed on PDA agar dishes. Any fungus growing out of the leaves that displayed a habitus similar to *A. alternatum* was checked microscopically and by PCR. *A. alternatum* was detected in older tomato leaves and confirmed by PCR but not in fruits or young leaves (data not shown).

2.3.5.2. Preparation of priming solutions of A. alternatum

Two priming solutions were tested in this work: a crude cell wall extract (CWE) of the fungus and a spore suspension, autoclaved for 20 minutes.

The CWE was prepared according to a protocol from the laboratory of Ralf Oelmüller (Institute of Plant Physiology, Jena, Vadassery et al. 2009).

Erlenmeyer flasks were prepared with 500 ml PDA and 500 µl of a spore suspension of *A. alternatum* was pipetted in the flask and kept on a rotary shaker in the dark for 3

weeks at 28°C. The CWE was prepared 3 times during this study and usually 6 l of PDA medium was used that was split up in roughly 20×500 ml Erlenmeyer flasks.

The mycelium was harvested by filtering through a nylon membrane and washing 3 to 4 times with sterile water. Then the mycelium was homogenised with pestle and mortar and the homogenate filtered through 3 layers of nylon membrane. The residue was homogenised again in sterile water 3 more times using pestle and mortar and filtered through the nylon membrane. After this the residue was homogenised 2 times in a mixture of chloroform and methanol (1:1) using pestle and mortar and filtered through the membrane. Again the residue was homogenised with acetone and filtered through the membrane 2 times. Then the residue was air dried under the sterile bench for at least half an hour.

The whitish powder that represents the mycelial cell wall was then suspended at 1 g per 100 ml in destilled water and autoclaved for 30 min at 121 °C. After cooling, the extract was filtered through 3 layers of nylon membrane, then 3 layers of filter paper and then filter strerilised using a 0.22 micron pore size filter. The filter sterilisation step was skipped for experiments on soil. This represented a 1% crude CWE from *A. alternatum*.

2.3.6. Fungus-plant interaction

2.3.6.1. Plate assay: Growth promotion and senescence

Arabidopsis mutants, defective in hormone signaling pathways, were grown for 2 weeks on agar plates with HL medium (pH 5.6) in the climate chamber (long day conditions; 23°C for 16 hours light, 18°C for 8 hours dark). Plants were then inoculated with *A. alternatum* by carefully pipetting 10 µl of a spore suspension (10^6 spores/ml) approximately 5 mm below the seed on the roots. A control group was treated with the same amount of sterile water. Ten to 14 days after treatment plants were rated according to their growth stage (Boyes et al. 2001). In some experiments the stem length of plants was measured. Roots from all plants were cut and the biomass of green parts instantly measured on a balance. The growth promotion (gp) was calculated from average fresh weights (afw) of the plants as

$$gp\,(\%) = \frac{afw\,(wildtype\,treated) - afw\,(wildtype\,control)}{afw\,(wildtype\,control)} \times 100,$$

this constitutes the growth increase in %.

Two hormone blockers were used in this study. The triazole fungicide propiconazole serves as brassinosteroid biosynthesis inhibitor in *Arabidopsis* (Hartwig et al. 2012). Seven-day-old Arabidopsis seedlings were treated with 10 µM propiconazole and inoculated with *A. alternatum* 7 days later and the biomass of plants was recorded 14 days after inoculation. Naphthylphthalamic acid (NPA) inhibits the polar auxin transport (Geldner et al.

2001). Ten µM NPA were added to Hoaglands medium before pouring of the plates and Arabidopsis plants were grown on these plates. Inoculation with *A. alternatum* was done 14 days after germination and the biomass was recorded 14 dai.

2.3.6.2. Plate assay to assess the biocontrol potential of the fungus

A desireable trait of beneficial fungi is their ability to facilitate plant growth.

So far *A. alternatum* is not known to produce any antagonistic enzymes. In this study it was tested on agar plates if the fungus can solubilise phosphate or fix nitrogen (=biofertilisation) or produces the antagonistic enzymes protease, chitinase or cellulase *in vitro* (Schwyn and Neilands 1987; Zheng et al. 2011; Gaiero et al. 2013). For this either a drop of the fungal spore suspension (10 μl containing 10^7spores/ml) or a piece of agar with the fungus on it was placed in the middle of the medium. Each experiment was done with 5 replicates and repeated once. The plates were cultivated at 26°C in an incubator and checked routinely for any signs of positive activity. The experiments were terminated after 2 months or earlier if a positive reaction was observed.

In most cases fungi known to produce the respective enzymes were co-cultured as positive controls on seperate plates.

The competitive potential of *A. alternatum* was tested in a fungus vs. fungus interaction assay on agar plates. For this an agar block with *A. alternatum* was grown on PDA for 1 week to make up for the slow growth of the endophyte in comparison to most of the fungi that this species was tested against. The opposing fungus was then placed on the same petri dish 4 cm apart from the *A. alternatum* colony. All plates were put in an incubator at 26°C in the dark and the experiment was terminated when the plate was overgrown with the interaction partners which took up to 3 months. The following phytopathogenic fungi were tested against *A. alternatum*: *A. alternatum*, *Aspergillus niger*, *Bjerkandera adusta*, *Cladosporium* spec., *Fusarium culmorum*, *F. avenaceum*, *F. graminearum*, *Gloeophyllum sepiarium*, *Macrophomina phaseolina*, *Talaromyces islandicus*, *Piriformospora indica*, *Schizophyllum commune, Trametes quercina* and *Trichoderma* spec..

2.3.7. Molecular methods and tools

2.3.7.1. DNA extraction

For reference DNA of rapeseed and Arabidopsis, seedlings were grown on agar plates with MS medium. Whole seedlings in the 2 to 4-leaf-state were used for DNA extraction.

The following protocol was used as standard for DNA extraction: up to 100 mg tissue was disrupted under liquid nitrogen using pestle and mortar, ground to a powder and transferred to a pre-cooled tube on ice. After this 700μl 2$\times$CTAB buffer with 7 µl 1% 2-mercaptoethanol was added and the tube was incubated at 65° C for 50 minutes. After a centrifuge step (16000 g, 10 min) the supernatant was transferred to a new tube and

incubated with 3 µl RNAse A (10 mg/ml) at 37° C for 30 minutes to degrade RNA. To the supernatant 0,7 ml chloroform/isoamylalcohol (24:1) was added, shaken for 3 minutes and centrifuged (18000 g, 20 min). The last step was repeated once. To the supernatant 1/10 volume 3 M sodium acetate (pH 5,2) and 1 volume isopropanol was added, mixed and kept at -20°C for 20 to 60 minutes or overnight for DNA precipitation. After another centrugation step at 18000 g (4° C, 25 min) the pellet was washed with 70% ethanol 2 times and centrifuged (10000 g, 25 min). After complete removal of the ethanol with a small pipette tip the pellet was dried at room temperature and then resuspended in 50 to 100 µl nuclease-free water. The DNA was stored at -20° C.

For the quantification of fungal and protist DNA from rapeseed 3 tissue types were used: root, hypocotyl and leaves. DNA was extracted with the innuPREP DNA Plant kit (Jenaanalytik) following the manufacturer´s instructions.

After extraction the DNA was quantified using the nanodrop spectrophotometer. The DNA solution was thoroughly mixed and 2 µl were used for each measurement, only samples with a 260/280 ratio of $\geq$1,8 were used for further studies.

2.3.7.2. RNA extraction from roots

Tissue intended for expression analysis was immediately processed after harvesting. The fresh biomass of the root samples was determined. The tissue was then immediately ground to a fine powder under liquid nitrogen using pestle and mortar. A sufficient amount of the RNA extraction reagent RNAzolRT (MRC) was added to 1 ml per 100 mg tissue, mixed carefully and then transferred into a fresh reaction vessel and processed according to the manufacturer´s protocol: to the homogenate 0,4 ml nuclease-free water was added, shaken vigorously for 15 seconds and stored for 15 minutes at room temperature. After centrifugation (12000 g, 15 min) 1 ml of the supernatant was transfered to a new tube and mixed with 0,4 ml 75% ethanol. The samples were stored for 10 min at room temperature or -20° C for 1 hour and then centrifuged (12000 g, 8 min). Next, the RNA pellet was washed twice with 0,4 ml 75% ethanol and centrifuged (8000 g, 3 min). The ethanol was removed with a small pipette and allowed to dry off for a short time. The RNA pellet was dissolved in nuclease-free water and an RNAseinhibitor such as Ribolock was added at a sufficient amount (1 μl/40 μl sample). RNA was stored at -80° C. For some experiments disrupted tissue was stored in RNazol at -80°C for up to 1 year as backup.

For rapeseed the RNAzol protocol did not work efficiently enough and the RNA yield and quality was low. Therefore, RNA from rapeseed roots was extracted with the RNA Plant easy mini kit (Qiagen), following the manufacturer´s instructions.

After extraction the RNA was quantified using the nanodrop spectrophotometer and 2 µl of carefully mixed RNA solution. After RNAzol extraction most samples contained residual DNA as indicated by a 260/280 ratio below 2,00. In these samples the DNA

was digested using the DNAse protocol from ThermoScientific: up to 1 µg RNA was added to a mixture of enzyme buffer, DNAse and RiboLock RNAse inhibitor to 50 µl and incubated at 37° C for 30 min. After this the digested RNA was either directly cleaned using the RNA Clean Up Kit (ThermoScientific) and following the manufacturer´s instructions or by the following protocol: after digestion the sample was incubated with 4 µl 25mM EDTA for 10 min at 65° C to deactivate the DNAse enzyme. Next, 1 volume phenol/chloroform/isoamylalcohol (25:24:1) was added to the sample, shaken and centrifuged (10000 g, 4° C, 10 min). The supernatant was carefully transfered to a new tube with 1/10 volume 3 M sodium acetate and 2 volumes ethanol absolute and stored at -20° C over night for precipitation of the RNA.

The next day, the sample was centrifuged (10000 g, 4° C, 20min) and washed twice with 70% ethanol, followed by another centrifugation step (10000 g, 4° C, 5 min). Residual ethanol was removed and the pellet air dried for some minutes to allow all traces of ethanol to evaporate. The pellet was then resuspended in nuclease-free water and measured on the nanodrop spectrophotometer.

2.3.7.3. RNA quality and integrity

The quality of each sample was checked with the nanodrop spectrophotometer and on an agarose gel or with the Bioanalyzer (Biorad). Only samples with sufficient quality were used for the expression analyses. For gelelectrophoresis a 2% SB gel (see section 2.3.7.5) was prepared and the samples were supplemented with loading buffer for RNA samples. Prior to loading the gel the samples were incubated at 65°C for 5 minutes to prevent the forming of secondary structures. After the run the gel was examined under UV for detection of the 2 distinct RNA bands: the 18S and the 28S RNA (Fig. 2.3).

The Bioanalyzer reports the integrity of the RNA samples which reflects how intact the RNA sample is. The scale ranges from 0 to 10 with 10 being the best integrity score achievable, values above 7 are considered still good. In this study most samples reached an integrity of 8 or above. Only samples with approximately the same integrity ($\pm 0,5$) were used for subsequent analyses.

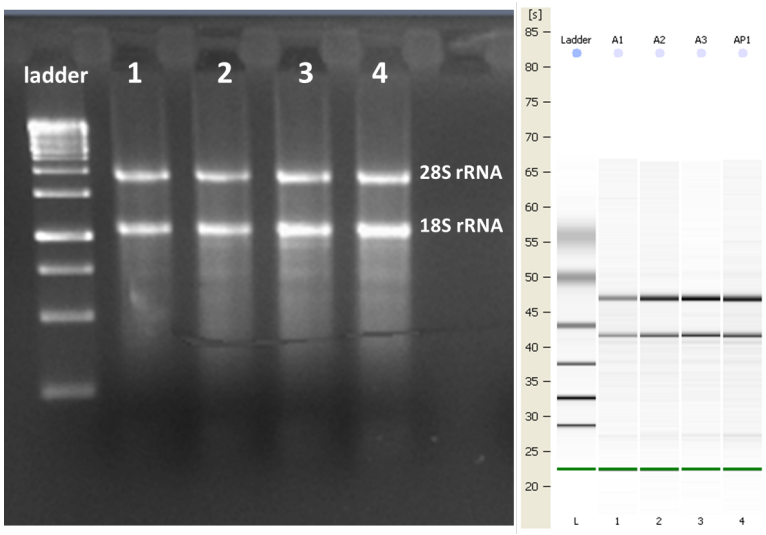


Figure 2.3.: RNA bands obtained after gelelectrophoresis (left) and with the Bioanalyzer (right). Shown are representative examples of DNase digested RNA samples from roots used for expression analyses. Note the 2 distinctive bands of the 28S and 18S rRNA displayed in both images.

2.3.7.4. cDNA synthesis

For semi-quantitative RT-PCR the Reverse Transcriptase from ThermoScientific was used following the manufacturers protocol. For RT-qPCR the Maxima First Strand Reverse Transcriptase (ThermoFisher) was used and the manufacturers protocol was followed.

According to Udvardi et al. (2008) the quality of the transcribed cDNA can be assessed by using 2 primer pairs for 1 reference gene that are at least 1 kb apart. Since the trancription begins at the 3´-end of the mRNA and does not always extend to the 5´-end of the template one would expect a higher Ct value of the primers at the 5´-end. A good measure for the efficiency of the cDNA synthesis and the respective quality of this cDNA is a Ct value difference between both primer sets of less than 1 cycle. For this study 2 primer pairs for the reference gene *EF2* were designed that are 2,3 kb apart. In all studies the difference between Ct values of both primers was less than 1 cycle, indicating that the cDNA used was of good quality.

2.3.7.5. Gel electrophoresis

At the beginning of this study, 500 to 1000 kb length fragments were run on 1,5%TAE or TBE gels. However, for smaller fragments such as the *A. alternatum* amplicon (258 bp) or genes for the qPCR studies the resolution of TAE and TBE gels were not sufficient. After staining in ethidium bromide bands below 1000 bp were unsharp and diffuse. It was therefore decided to use the alternative buffer SB (*S*odium *B*oric acid buffer; Brody and Kern 2004b,a; Brody et al. 2004). A 10 fold stock solution was prepared by dissolving 100 mM sodium hydroxide in destilled water and adjusting the pH of the solution to 8,5 with boric acid. This solution was diluted 1:10 to get a 1 fold working solution. This buffer

worked very well for fragment sizes of 90 to 1000 bp and was therefore used for all other applications as well. Another advantage is the low heat conductivity of the buffer that allows a higher voltage to be applied and therefore a faster run. Usually 2% agarose gels were used for gel electrophoresis.

2.3.7.6. Candidate genes for studies on defence mechanisms in Arabidopsis

At the beginning of the study a list of genes was compiled of potential candidate genes involved in resistance mechanisms (Table 2.4). Most of these genes had shown an interesting behaviour in a previous study (Siemens et al. (2006)). In this study a microarray was carried out from Arabidopsis roots previously inoculated with *P. brassicae* and harvested 10 dai and 23 dai.

Figure 2.4.: Candidate genes of Arabidopsis for resistance pathways potentially influenced by *P. brassicae* and / or *A. alternatum*. Genes were choosen according to their regulation in the root microarray from Siemens et al. (2006). For upregulation the cut-off value is $\geq$2, for downregulation it is $\leq$0,5. Regulation was calculated as signal intensity (treated) / signal intensity (control). Abbreviations: ET - ethylene, JA - jasmonate, SA - salicylic acid, SAR - systemic acquired resistance.

Gene	Description	Annotation	Function	Relative expression in the microarray from Siemens et al. (2006)	
				10 dai	23 dai
COI1	coronatine insensitive 1	AT2G39940	constitutes SCF ubiquitin ligase complex, required for JA-induced regulation	1,79	1,14
ETR1	ethylene response 1	AT1G66340	ethylene receptor	0,98	1,35
GH3.5	Gretchen Hagen 3-5	AT4G27260	IAA-amido synthase conjugates amino acids to auxin	0,74	5,55
JAR1	jasmonate	AT2G46370	catalyses formation of JA-isoleucin	0,50	0,45
NPR1	nonexpressor of pathogenesis-related genes 1	AT1G64280	key regulator of SAR, confers resistance to several pathogens	0,51	0,63
PDF1.2	plant defensin1.2	AT5G44420	JA marker gene, ET and JA-responsive plant defensin	8,14	3,13
PR1	pathogenesis-related gene 1	AT4g33720	SAR marker, induced by a variety of pathogens	5,26	0,09
SID2	salicylic acid induction deficient 2	AT1G74710	isochorismate synthase, involved in SA accumulation	1,18	0,97
THI2.1	thionin 2.1	AT1G72260	JA marker	2,46	93,48

Another set of genes was chosen to study the early interaction of both plant pathogens in *Arabidopsis* roots. These genes are reportedly involved in the recognition of microbes (*BAK1, FLS2*; Dodds and Rathjen 2010) or in early pathways that lead to induction of resistance (*WRKYs, MAP, MYBs, CYP*s; Millet et al. 2010).

Table 2.8.: *Arabidopsis* genes involved in early resistance pathways.

Gene	Description	Annotation	Function
BAK1	BRI1-associated receptor kinase	AT4G33430	component of the brassinosteroid receptor
CYP71A12	cytochrome p450	AT2G30750	involved in camalexin biosynthesis
FLS2	flagellin-sensitive 2	AT5G46330	protein kinase, involved in MAP kinase signaling and innate immunity
MAP	membrane associated protein family member	AT5G25260	nodulin-like protein, function unknown
MYB51	myb domain protein 51	AT1G18570	activates promotors of glucosinolate biosynthetic genes
WRKY11	WRKY DNA-binding protein 11	AT4G31550	negative regulator of basal resistance in Arabidopsis
WRKY18	WRKY DNA-binding protein 18	At4G31800	pathogen-induced transcription factor

2.3.7.7. Resistance genes in Brassica

For *B. napus* it was planned to test the same genes as those chosen for Arabidopsis. At the beginning of this study (2010) this seemed an achieveable task as it was predicted that the genome of rapeseed would be available in 2011. Until then mainly QTLs and a handful of resistance genes had been published for rapeseed. Breeders working on resistant *Brassica* cultivars rely mainly on data from seed companies that are not publicy available. In 2012 it was estimated that a first draft of the *B. napus* genome would be published in 2013 (D. Edwards, pers. communication). However, only after the experimental work for this project was finished in 2014 the complete sequence of *B. napus* was published which was too late for further studies for this work (Chalhoub et al. 2014).

The main problem is that most of the resistance genes in Arabidopsis are not homologous to *Brassica* and that no tools were available online that worked well on predicting homology between Arabidopsis and *Brassica*. Hence, primer pairs for reference and resistance genes from the literature were tested and used when the PCR results were correct. The list of utilised genes from *B. napus* is shown in table 2.9.

Table 2.9.: Published genes from *B. napus* that were used in this study and their functions according to the literature (listed under "Source"). Abbreviations: JA - jasmonate, MAMP - microbe-associated molecular pattern, SA - salicylic acid.

Gene	Description	Annotation	Function	Source
BnWRKY33	WRKY DNA binding protein 33	KF712488	activates SA- and JA-responsive genes, MAMP-response	Wang et al. (2014); Lloyd et al. (2014)
BnPR1	pathogenesis-related gene 1	AY623008	SA-responsive gene	Wang et al. (2012, 2014)
BnPDF1.2	plant defensin 2.1	AY884023	JA-responsive gene	Wang et al. (2012); Wang et al. (2014)
BnMPK3	mitogen-activated protein kinase 3	AY642433	activates SA- and JA-responsive genes, MAMP-response	Zhao et al. (2009); Lloyd et al. (2014)
BnMPK4	mitogen-activated protein kinase 4	EU581868	SA-JA-crosstalk gene, MAMP-response	Wang et al. (2012); Lloyd et al. (2014); Wang et al. (2014)
BnPEP	phosphoenolpyruvate carboxylase	D13987	reference gene	Wu et al. (2010)

2.3.7.8. Quantifying relative gene expression

With the relative quantification the change in transcript levels of a gene of interest (GOI) in relation to a control group is determined (Livak and Schmittgen (2001)). For this mRNA of the investigated sample is extracted and transcribed to cDNA. Because the amount of cDNA cannot be measured on the spectrometer due to residual nucleid acids after transcription it is later normalised in the qPCR experiments by the use of 1 or more reference genes. Ideally, the expression of a suitable reference gene is not influenced by the treatment itself.

2.3.7.9. Selection of reference genes for normalisation

Reference genes were selected on the basis of their stable expression during *P. brassicae* development in Arabidopsis. A microarray conducted by Siemens et al. (2006) provides information of gene expression at the 2 time points 10 and 23 dai. A list of genes whose expression signals did not vary at both time points during both treatments - control and infected - was chosen for further evaluation. Using the tool Genevestigator (https://www.genevestigator.com/gv/plant.jsp, last accessed in July 2014) and AtGenex-

press (http://jsp.weigelworld.org/expviz/expviz.jsp, last accessed in July 2014) the stability of expression under various stress conditions was examined. Three genes with different expression levels - matching the range of expression of the GOIs - resulted from this survey. The 3 genes encode for an elongation factor (*EF2*), the yellow leaf specific protein 8 (*YLS8*) that is involved in mitosis and a vacuolar ATPase (*VATP*). Furthermore, using the Excel(R) macro NormFinder (Andersen et al. 2004) all genes were tested regarding their stable expression under the experimental conditions with 4 treatments, 3 of them being inoculation with pathogens. The stable expression of all reference genes could later also be confirmed in the microarray conducted 3 dai with the treatments used in this project (data not shown).

2.3.7.10. Primer design

Except when indicated otherwise, all primers used in this work were designed by the author using the online tool Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/; last accessed in May 2015). Settings were used as pre-installed except for the following: maximum 3´ self-complimentary (set to 0), maximum repeats of one base ("Max poly-x" = set to 4), desired primer size (set to 25 bp), annealing temperature (set to 60°C) and a desired product length from 500 to 1000 bp for semi-quantitative PCR and 100 to 250 bp for real time PCR.

Primers for *B. napus* were obtained from the literature. The complete list of all genes and primers utilized in this study is depicted in table 2.10.

All primers were ordered desalted, for qPCR reactions primers were ordered to be cleaned by High Performance Liquid Chromatography.

Table 2.10.: Complete list of all genes investigated in this study and their corresponding primer sequences. Annealing temperature for all primers is 60°C except for genes with an asterisk (*) it is 62°C. The gene *CYP71A12* cannot be distinguished from *CYP71A13* in a microarray. If not indicated otherwise all primers were designed by the author with the software Primer3Plus.

Name	Forward Primer	Reverse Primer	Amplicon length	Source
Arabidopsis thaliana				
BAK1	TTGAATGTTGTTGAAAGCTAAAACC	GATAACACAGAAACCGGTAAGAAA A	147	
COI1	TTGGGGTTTCCACAAACTGT	TAAAGTAAACAGACCCCTGAGGAAA	152	
CYP71A12 /13	GTTATGAAGAAACTCCAAGACGAGA	ATCTGGGAAGTATTAGTGGAAGAGG	154	
EF2A	CACTCTTACTGATTCTTTGGTTGCT	TAAGATTGATGAGGTACTCGTTTCC	209	
EF2B	TACTCTTATGGTATGACGGATTGTG	ATATGAATGATCGGAAGAGAAAAGA	166	
*ETR1**	AGCTGATGTTCTCTGGTTTAATTGT	ATTATACCACCACCATCTTGTTCCT	159	
FLS2	TGCTTATATGAGGAAAGTGACAACA	GAGTTCCATATCAAGAACCCTAACC	193	
*GH3.5**	GACTCTCTTTGAGCTAGAAGGTTTG	GAGAAAGAGTGATGAGAGTTGGTTG	194	
*JAR1**	GACAGTTTAAGATGCCAAGATGTG	ACAACGACGGAAATAAACAACTAA A	161	
MAP	CTCGATACAGTTTACGAGCAGA	ACCTCAAACACTTACGAATAAACCA	196	
MYB51	ATGATGTTGGAGGAGTCTTGTGT	GTGGGATTCATCGATTATGAGTTT	177	
NPR1	CGTCATATAGTTTCGCTCTTCGT	ACACCATCATGGGTTATTGTTG	144	
PDF1.2	ATTAACCTTGAAGGAGCCAAACA	TTGTAACAACAACGGGAAAATAAAC	207	
PR1	CGGTCCGGTCTTCTTTTGT	AAGCCGTAGCATTGCATTTT	83	
SID2	AAGATATCTCAGGTACGAGCTTTTG	AATTAATCGCCTGTAGAGATGTTGT	159	
THI2.1	TACGTGTCTTTTATTGACCTTCCTC	GAGTTTCTGGATCCTTAAGTTCGTT	155	
VATP	TGGACATTGCTCCGTATCTTC	TCGATAAGATAACCTCCATTACCTC	121	
WRKY11	AAACCGATCAAGGGCTCAC	CTGGATCATCTAATGCTCGTTC	103	
YLS8	TTGGTGATTGCTCCAAAAGA	AAATGGAGAACAACCGAAACA	197	
Brassica napus				
BnMPK3.1	GATCTAAAGCCTAGCAATCTTCTCC	ATGAGCTCCATAAAGATACAACCAA	209	
BnPEP	CAGTTCTTGGAGCCGCTTGAG	TGACGGATGTCGAGCTTCACA	140	Wu et al. (2010)
BnPDF1.2	CATCACCCTTCTCTTCGCTGC	ATGTCCCACTTGACCTCTCGC	193	Wang et al. (2012)
BnPR1	CCTTCACTATACTCAGGTTGTTTGG	ATTGCACGTGTTTTATATGTCGATG	173	
BnWRKY33	ATCCCAAACTTTAGATCTTTTGCTC	TGTTGTTGTTGTTGTTCTTCTCTTC	105	
Acremonium alternatum				
OLG	GCCCGCTCCCCCGGAAG	GCCCGCTCCCCCGGAAG	258	Jäschke et al. (2010)
Plasmodiophora brassicae				
Pb_Cao	GTGGTCGAATTCATTAAATTTG GGCTCT T	TCAGCACCGTTTCCGGCTGCT AAGGC	150	Cao et al. (2011)

2.3.7.11. Standard PCR and semi-quantitative RT-PCR

To test new primer pairs standard PCR was carried out. Each single reaction was done in a 20 µl reaction volume. Two different polymerases were used: either 0,5 µl Taq polymerase (2,5 u/µl), self cleaned up from the laboratory Ludwig-Müller with 1 µl $MgCl_2$ (2,5 mM) and 2 µl buffer or 0,1 µl DreamTaq (5 u/µl; ThermoFisher) with 2 µl DreamTaq buffer. Each tube contained 1 of the 2 Taq polymerases, 1 µl of each primer (10 pmol), 1 µl loading dye, 2 µl dNTPs, 2 µl template DNA and the respective amount of nuclease-free water to amount to 20 µl.

As template or positive control genomic DNA (25 ng/µl) of the respective organism was used. A master mix was prepared for all samples with 1 additional sample volume per 10 reactions.

An Eppendorf mastercycler epgradientS was used for standard PCR. If not indicated otherwise the following PCR program was used: 2 min 94°C, 30 cyles with 30 sec 94°C, 30 sec 60°C and 2 min 72°C, followed by a final elongation step at 72°C for 5 min and the break at 10°C

To find the optimum annealing temperature a temperature gradient was programmed in the cycler that ranged from 54 to 64 °C. The temperature that yielded the thickest band on an agarose gel was determined as optimal annealing temperature (60°C for most primers).

Semi-quantitative RT-PCR was done with cDNA transcribed from the RNA samples. The reaction volume and setup was the same as described above. The cDNA template was diluted 1:10 as stock solution and then further diluted as needed. Initial semi-quantitative experiments started with a 1:100 dilution of each cDNA. cDNA of all samples from 1 experiment was amplified with the reference gene *YLS8* and the PCR products were run on an agarose gel. Depending on the intensity of each band on the gel the cDNA was either further diluted or a higher amount of cDNA was used in the following PCR. This procedure was repeated until all bands showed more or less the same intensity on the gel. The so obtained working concentrations of cDNA were checked with the 2 additional reference genes *EF2* and *VATP*. The working concentration for gene expression analysis ranged for most genes from 1:100 to 1:1000. For *PDF1.2* and *THI2.1* no product could be amplified with a 1:100 dilution in most experiments. For these genes a dilution of 1:5 to 1:25 and up to 40 cycles were used.

In general, sqRT-PCR has several drawbacks. The reference gene must be carefully chosen as normalisation to more than 1 reference genes is not practiable with this method. In addition, the interpretation of sqPCRs is highly subjective and depends on many factors, e. g. the number of PCR cycles used, the effectiveness of the DNA stain ethidiumbromide, the resolution of the gel picture, the quality of the cDNA and a representative control sample. It was observed that thawing and refreezing of cDNA samples resulted in a lower

signal intensity over time which is another factor to be considered when comparing band intensities on a gel. Therefore analyses with the much more sensitive qPCR (see next section) were favoured in this study.

2.3.7.12. qPCR data analysis

Quantitative PCR relies on the detection of a fluorescence signal of the double strand DNA binding dye SYBR Green. The dye binds to the PCR product and emits a fluorescence signal. This signal is detected only upon cleavage by a detector and analysed with the software of the qPCR cycler. This principle is based on the fluorescence resonance energy transfer (Giulietti et al. 2001). The intensity of the fluorescence is directly proportional to the amount of amplificate produced, thus giving back the quantity of transcript of the used cDNA. The accumulation of PCR product is exponential because it is an enzymatic process and can be described by a sigmoidal curve with the number of cycles at the x-axis. In the early PCR phase only small amounts of PCR product are amplified that cannot be seen because of the level of background fluorescence. As the PCR continues, larger amounts of product are produced and the fluorescence intensity increases until it rises above the background and becomes visible. This is the point at which the software measures the crossing point or Ct value (=threshold cycle number).

A protocol was established for the laboratory that addresses all key components of reliable qPCR experiments (Vandesompele et al. 2002; Fleige et al. 2006; Yuan et al. 2006; Udvardi et al. 2008; Bustin et al. 2009, 2010; Derveaux et al. 2010).

According to the literature each PCR run should contain: standards of all genes investigated in this run, at least 1 reference gene, as many genes of interest as possible, samples of all treatment groups from one time point of harvest and no-template-controls to confirm no contaminations were present in the PCR master mix (Vandesompele et al. 2002; Yuan et al. 2006; Udvardi et al. 2008; Bustin et al. 2010). Following this protocol run-to-run variation could be assessed and the PCR efficiency calculated after each run.

Each experiment was pipetted on ice and SYBR Green containing reagents were kept in the dark to prevent degradation of reagents prior to the reaction. In each run the complete 96 well plate of the cycler was used and a pattern for probes was set up that was applied in each experiment. Normally, 3 genes of interest and 1 reference gene including the standards for all 4 genes were investigated in 1 qPCR run. For most of the experiments a melting curve analysis was done after the last cycle. For this the cycler heats up the samples in 0,5 °C-steps to 90°C. The graphical display then shows a typical melting curve with 1 peak, representing the specific melting point of the amplificate.

Usually, each run was repeated 2 times and the following criteria were checked:

- "No template control" is negative (no Ct or Ct at least 5 cycles apart from the highest specific Ct)

- Ct within linear range of standard curve
- PCR efficiency 80 - 110 %
- $\triangle$ Ct of replicated GOI < 0,5
- positive controls are positive
- one specific peak at the melting curve

Only samples that met these criteria were used for further analyses. Relative gene expression was calculated by hand using the $\triangle\triangle$Ct-method (see next section).

2.3.7.13. Calculation of gene expression

To be able to calculate the relative expression of genes, the PCR data must be normalised. First, normalisation of Ct values from treated samples to the control samples is done in order to take into account the growing conditions of the plants and "substract" any effects that could result from the specific growing period. Second, since cDNA values cannot be measured directly, the data must further be normalised to 2 or 3 internal reference genes, thus normalising basically to the amount of cells and substracting effects that result from a varying amount of inserted cDNA in the experiment.

Expression values were calculated by hand using the $\triangle\triangle$Ct-method (Winer et al. 1999). One assumption of this method is that the amplification efficiency of the target and reference genes is approximately equal (Livak and Schmittgen 2001). Thus, the PCR efficiency was determined for all qPCR experiments by use of a standard curve for each gene of interest (GOI) and the so determined efficiency was included in the formula for gene expression with the term $1 + efficiency^{\Delta\Delta Ct}$(Schmittgen and Livak 2008).

Only Ct values of successful qPCR assays that met the quality criteria described above (section 2.3.7.12) were used for the calculation of relative gene expression. Each sample was run as triplicate and each PCR was repeated twice.

For each experiment 2 to 3 reference genes were used for normalisation of the cDNA values. After all PCRs were done for each time point and treatment, the Ct values for all tested reference genes were pooled, normalised and averaged so that the PCR data from all GOI could be normalised to 1 pooled reference gene-value for each treatment.

The calculation was done as follows:

1. Calculate the arithmetic mean of all valid Ct values of each gene from 1 PCR experiment, here termed as $\overline{Ct}$;
2. Normalise the $\overline{Ct}_{treated}$ to $\overline{Ct}_{control}$ by $Norm_{\overline{Ct}} = 1+efficiency^{(-(\overline{Ct}_{treated}-\overline{Ct}_{control}))}$;
3. Calculate the geometric mean $Geomean_{ref}$ of $Norm_{\overline{Ct}ref}$ of all reference genes from all PCRs (Vandesompele et al. 2002);

4. Calculate the relative gene expression of each GOI (RE_{GOI}) by calculating the ratio $RE_{GOI} = \frac{Norm_{\overline{Ct}GOI}}{Geomean_{ref}}$, this represents the raw expression data.
5. Log-transform the data by LOG2 ($LOG2_{RE}$) with $LOG2_{RE} = LOG\,RE_2$; this represents the transformed relative gene expression data of 1 PCR experiment.
6. Calculate the arithmetic mean of $LOG2_{RE}$ for all 3 PCRs for each sample; this represents the transformed relative gene expression data for each experiment.
7. Calculate the standard deviation from the 3 $LOG2_{RE}$ values of each PCR.

2.3.7.14. Quantification of A. alternatum and P. brassicae from rapeseed roots

Rapeseed plants of the variety "Ability" were grown on soil under standard conditions in the greenhouse for 5 days and then inoculated with a spore supension described in section 2.3.2 with a concentration of 2×10^6 spores/ml. Fourteen days after treatment 6 plants per treatment were harvested and washed for several minutes with tap water.

Plants were then divided in root, hypocotyl and leaf tissue and frozen with liquid nitrogen in individual packages and stored at -80°C. Each package represented 1 biological replicate.

The DNA from 2 replicates of each tissue group was extracted using the innuPREP DNA kit (Jenaanalytik) and following the manufacturer´s protocol with the following changes: from the 3 available buffers the SLS buffer was used, RNA was digested with RNAse I (100 µg/sample) and the samples were eluted 2 times with 100 µl elution buffer.

The concentration and purity of the DNA was checked with the nanodrop spectrophotometer.

A dilution series was run on the qPCR cycler using 10 ng, 5 ng, 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 0,5 pg and 0,1 pg of *A. alternatum* and *P. brassicae* genomic DNA and the primer pairs specific for the respective organism (Table 2.10).

The standard curve was repeated once with each standard DNA dilution as quadruple in each 96-well plate. The standard curves were reproducible for both organisms (data not shown). The amount of pathogen DNA in each unknown sample was calculated with the equation from the regression curve (y=ax+b):

1. $log\,x = (Ct_{sample} - b)/a$
2. $amount_{PCR} = 10^{log\,x}$
3. $\frac{(amount_{DNA} \times dilution\ factor)}{fresh\ weight\ [mg]} = amount_{DNA}\ per\ mg\ fresh\ weight$

2.3.7.15. Microarray analysis

To determine the level of resistance genes at a very early stage in the infection progress a microarray (Agilent, 60k) was conducted. Agarwal et al. (2011) found in their Affymet-

rix microarray from clubroot infected Arabidopsis roots that the greatest transcriptome changes occured 4 dai (compared to 7 and 10 dai). Microscopy revealed that around 3 to 4 days after contact with fresh root hairs *P. brassicae* spores germinate and infect the root hairs (Ingram and Tommerup 1972). There are no microarray data available for Arabidopsis Col-0 inoculated with *P. brassicae* at an earlier time point than 4 dai. Additionally, microarray data for the inoculation with *A. alternatum* are lacking completely. At 3 dai the fungus *A. alternatum* is already established in the root (own results), hence the early time point 3 dai was chosen for this microarray.

The company Oaklabs, Berlin, carrying out the microarray requested biological triplicates for each treatment group and a high quality of each sample to ensure appropriate results. Quality parameters were defined as a 260/280 ratio of at least 2,0 and a 260/230 ratio of at least 1,8.

For this roughly 400 *Arabidopsis* Col-0 plants were grown hydroponically under the same conditions as described above (2.3.1.3) and inoculated (see 2.3.2). Not all seeds germinated and some plants died during the initial 2 weeks of growing which resulted in approximately 90 viable plants per treatment. Before harvesting each treatment group was divided in 3 equal parts, each containing approximately the same amount of plants in similar growth stages. This was done to ensure the equality of samples within each treatment group.

Roots were harvested 72 hours after inoculation, immediately ground in liquid nitrogen and frozen in RNAzol. RNA was then extracted from all samples and processed according to the manufacturer´s protocol, although most steps were performed on ice and not at room temperature. RNA samples that met the quality standards of the company were then packed and shipped on dry ice to Oaklabs, Berlin.

Each biological replicate consisted of 28 to 31 individual root samples. The company that handled the microarray did a t-test (equal variances, independent samples) for each treatment by comparing the means of the control group with the means of the respective treatment group. They also calculated the relative expression for each gene and treatment relative to the controls. They then log-transformed the results. Statistically significant results with $p<0,05$ were grouped together in one file that included the ATG-number, treatment and LOG2-transformed relative expression of the respective genes. The so grouped files were used for all subsequent analyses. The microarray contained 31844 alleles, including cross hybridisations and pseudogenes.

Upon retrieval of the data the software tool Mapman (Thimm et al. 2004) was used to get a better insight of the data. Mapman displays a graphic overview over large data sets and groups genes according to their function in pathways such as "Photosynthesis", "Secondary metabolites" or "Biotic stress". The resulting overview contains genes, represented as squares, that are involved in the particular pathway chosen, and their respective behaviour towards the treatment. For this study, upregulated genes were displayed

as red squares whereas downregulated genes wer in blue. The user can then see at one glance in which pathway most of the transcriptome changes occur and can find out which genes are involved specifically by clicking on the squares. This simplifies the handling of huge datasets like a microarray and provides a good basis to start subsequent analyses.

2.3.8. Histological methods

2.3.8.1. Light microscopy

Roots from endophyte-inoculated Arabidopsis seedlings were harvested and boiled in 10% KOH for 3 minutes, washed with tap water several times and stained in boiling 5% ink-in-vinegar for another 3 minutes. Destaining was done for 3 minutes in normal tap water (Vierheilig et al. 1998, 2005). This easy and cheap method was very efficient in making fungal hyphae visible under the light microscope in fresh root samples.

To observe the direct interaction between fungus and plant, root samples of *B. napus* roots were stored in 70% ethanol after disease rating until use. The root samples were fixed and embedded in glycolmethacrylate according to the manufacturer's protocol for Technovit (Heraeus Kulzer).

The Technovit blocks containing the samples were sliced with a microtome (Leica) in 5 to 7 µm thick slices, fixed on glass slides and dried on a baking plate at 40°C for at least 1 hour. Slides were stained after the method of Marques et al. (2013): 20 minutes staining in 5% lactophenol blue followed by 3 1-minute washes in distilled water. Counterstaining was done immediately after this with 0,2 to 0,5% aqueous safranin O followed by another 3 1-minute washes in distilled water. Slides were then dried at 40°C.

Cotton blue stains fungal hyphae, chitin and plasmodia while safranin stains plant cell walls (Marques et al. 2013). To test whether fungal hyphae of *A. alternatum* are stained with this method, some pieces of mycelium were scratched from a plate containing the fungus and fixed in fixation solution and then embedded as described above.

2.3.8.2. Scanning electron microscopy

Scanning electron microscopy was done with a ZEISS Supra 40VP microscope with SE detector. Fresh samples were put on a copper block and fixed on the block with Tissue-Tek(R) before deep freezing in liquid nitrogen under vacuum for half a minute. The frozen sample was then transferred to the cryopreparation unit (Emitech K1250X) and sublimed for 30 minutes before breaking. After coating with platin the broken sample was quickly transferred to the microscope and examined. The preparation of samples for the scanning electron microscope was done by Markus Günther, Dresden.

3. Results

Arabidopsis and rapeseed plants were inoculated with *A. alternatum* and *P. brassicae* according to the objectives (section 1.6; for inoculation see section 2.3.2.1) and the successful colonisation with both microorganisms was checked microscopically, by PCR amplification of organism-specific fragments and with a disease rating for the clubroot pathogen several weeks after inoculation with *P. brassicae*. The majority of clubroot-inoculated plants showed typical disease symptoms (see section 3.2). Further, gene expression of resistance genes was analysed using RT-PCR on transcribed cDNA from root tissue of inoculated samples (section 3.3). The broader biocontrol potential of *A. alternatum* was assessed by *in vitro* experiments.

3.1. Detection of A. alternatum and P. brassicae in plant tissues

To locate the fungus *A. alternatum* and the protist *P. brassicae* within plant cells light microscopy and scanning electron microscopy was done. It was expected to find the fungus within the intercellular space and possibly within the cells of root tissue and later (1 to 2 weeks after inoculation) in stems and leaves as well. *P. brassicae* completes its life cycle within the roots and is strictly soil-borne so that only roots were investigated for the presence of the protist.

Additionally, DNA from inoculated plant tissues was extracted and examined with PCR for the presence of *A. alternatum* and *P. brassicae*. For rapeseed an absolute quantification for both organisms was done with qPCR.

3.1.1. Light microscopy

In fresh root samples of endophyte-inoculated Arabidopsis fungal hyphae were detected in and around the roots using the ink and vinegar method of Vierheilig et al. (1998) that stains fungal hyphae blue and accentuates the contrast of the examined structures. Since here whole roots were examined the exact position inside the roots and the point of entrance in the root could not be determined exactly (see Fig. 3.1).

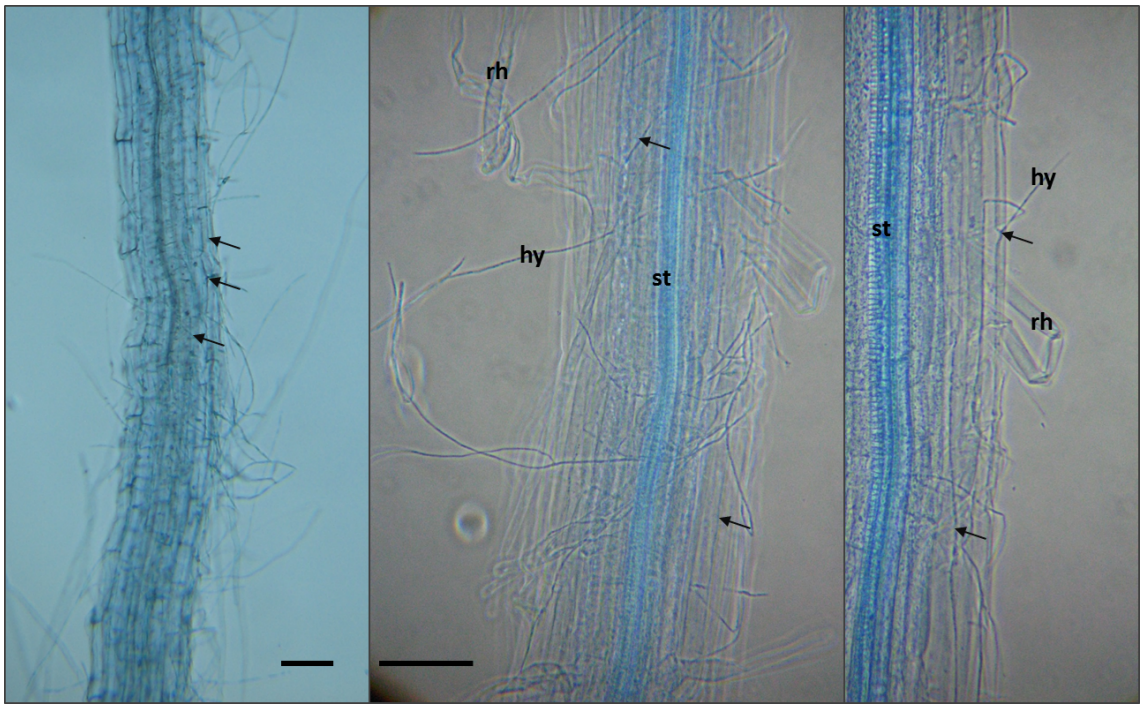


Figure 3.1.: Light microscopy images of Arabidopsis roots inoculated with *A. alternatum* 5 dai stained with 5% ink-in-vinegar (Vierheilig et al. 1998). Arrows indicate hyphae which are possibly located in the intercellular space of root cells. Abbreviations: rh - root hair, st - stele, hy - hyphae, dai - days after inoculation. Scale bars are 50 μm.

Samples of disease rated *B. napus* roots (var. Ability, var. Visby) and of respective control roots were prepared in Technovit and sliced with a microtome in 5 to 7 µm thick slices that represent 1 to 2 cell layers. The samples were stained with cotton blue to stain hyphae and plasmodia and counter-stained with safranin O to visualize plant cell walls (Marques et al. 2013; Fig. 3.2).

Hyphae from *A. alternatum* agar plates were stained in the same way as a positive control for the fungus. In some of the root samples, blue structures that resemble fungal hyphae were detected. However, it cannot be stated with absolute certainty that these structures represent hyphae and are not an artefact of the staining method (Fig. 3.2).

All root samples inoculated with *P. brassicae* showed the typical aberrations in cell organisation with hypertrophied cells that contained resting spores (Fig. 3.3). All roots from disease class 3 and 4 harboured resting spores of *P. brassicae*. Samples co-inoculated with *A. alternatum* showed similar structures (Fig. 3.3).

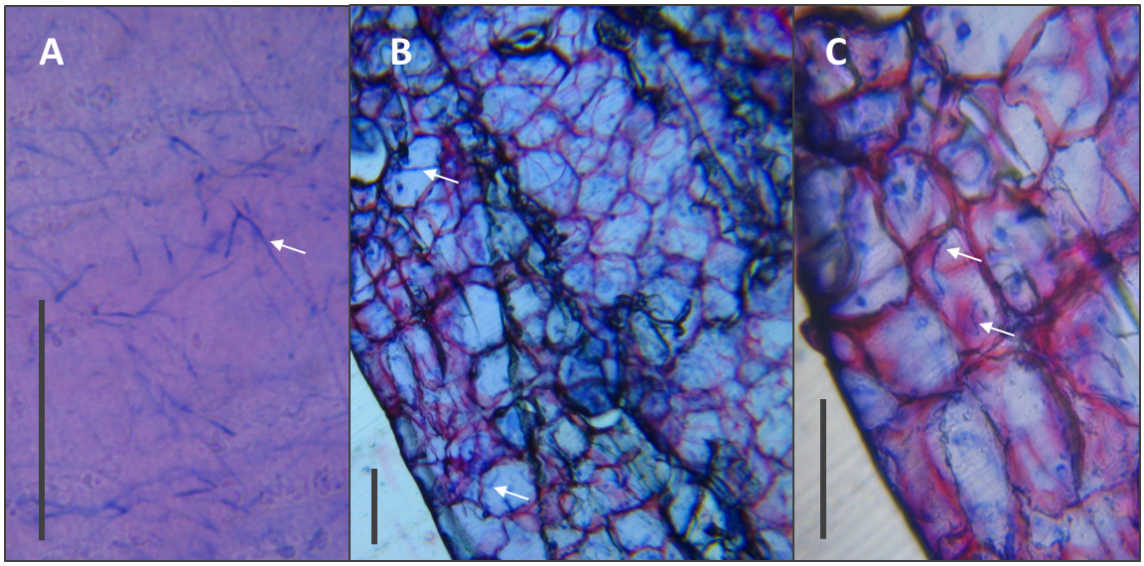


Figure 3.2.: Light microscopy images of *A. alternatum* (A) and root tissue from *B. napus* (B, C) 6 to 8 weeks after inoculation with *A. alternatum* stained with cotton blue and safranin O. Hyphae of the endophyte show a distinctive blue colour (A). Note the fungal-like structures within cells and in the intercellular space of root tissue indicated by arrows (B, C). The blue colour might be an artefact of the staining method and could not be detected in all of the examined samples (n=3). Scale bars indicate 10 μm.

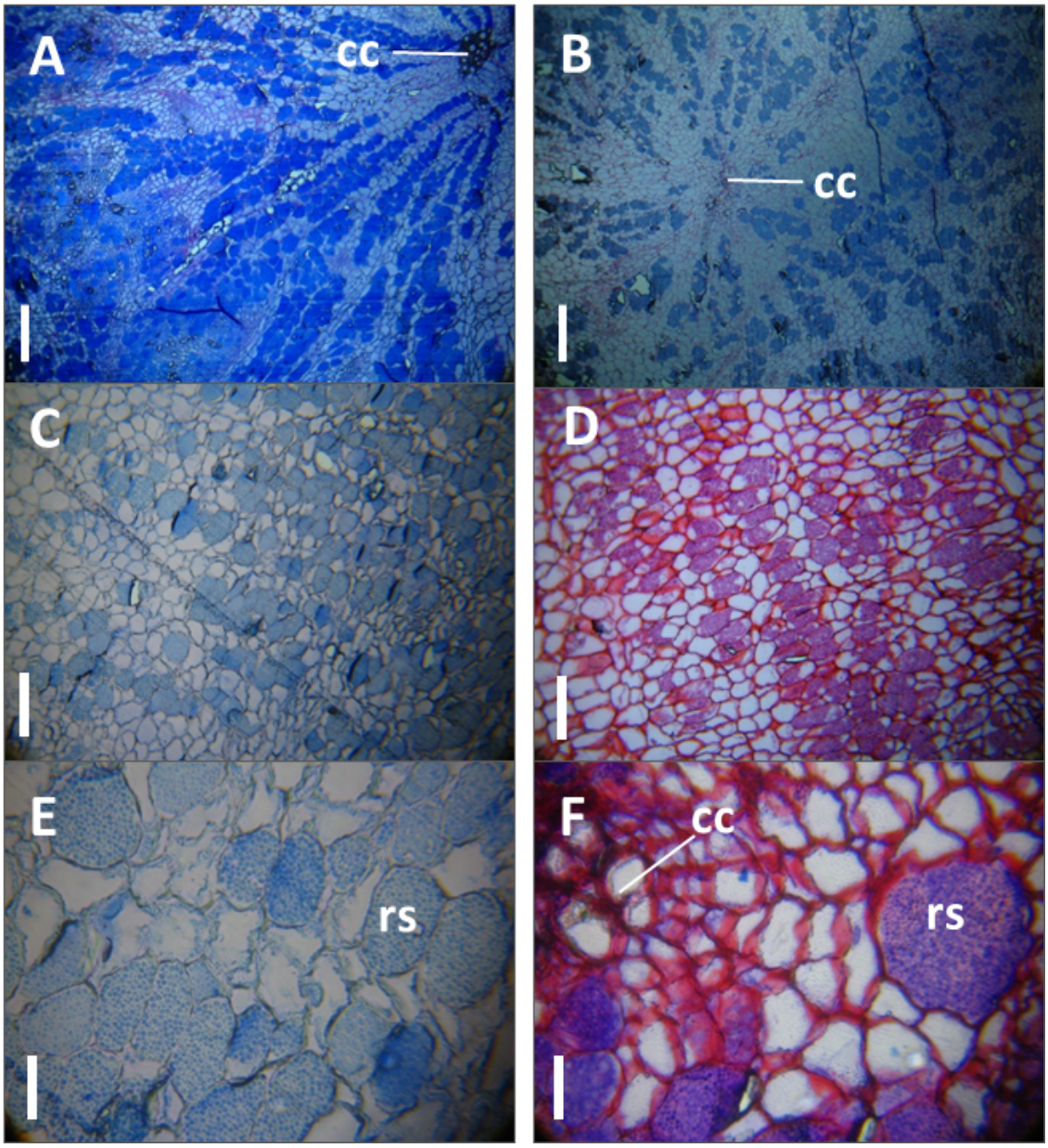


Figure 3.3.: Light microscopy images of *B. napus* roots 6 to 8 weeks after inoculation with *A. alternatum* and/or *P. brassicae* stained with cotton blue and safranin O. Co-inoculation with *A. alternatum* did not decrease the amount of resting spores visibly (left row) in comparison to roots treated with *P. brassicae* only (right row). Abbreviations: cc - central cylinder, rs - resting spores. Scale bars are 50 μm (A, B), 20 μm(C, D) and 10 μm (E, F).

3.1.2. Scanning electron Microscopy

Tissue samples from roots and leaves of *B. napus* var. Visby, inoculated with *A. alternatum* and *P. brassicae,* were examined with a scanning electron microscope (SEM) 9 weeks after inoculation. For this fresh tissue was fixed with cryopreparation as described in the methods section (2.3.8). Inoculation with the endophyte alone did not result in any obvious changes in the cellular organisation of root or leaf tissue (Fig. 3.4, right row). Unfortunately, no fungal hyphae could be observed in the analysed rapeseed tissues or

on the surface of the leaf with the SEM.

Root tissue of the rapeseed var. Visby inoculated with *P.brassicae* showed the typical structures distinctive for the clubroot pathogen under the SEM: plasmodia and resting spores within enlarged cells were clearly visible (Fig. 3.5) that were absent from samples without clubroot inoculation (Fig. 3.4). Co-inoculation with *A. alternatum* did not have a strikingly positive effect on the cellular organisation of the rapeseed roots (Fig. 3.5 A - D). Here as well enlarged cells containing plasmodia and resting spores were observed, indicating that resting spore formation was in progress. The exact ratio of plasmodia to resting spores was not determined in this study.

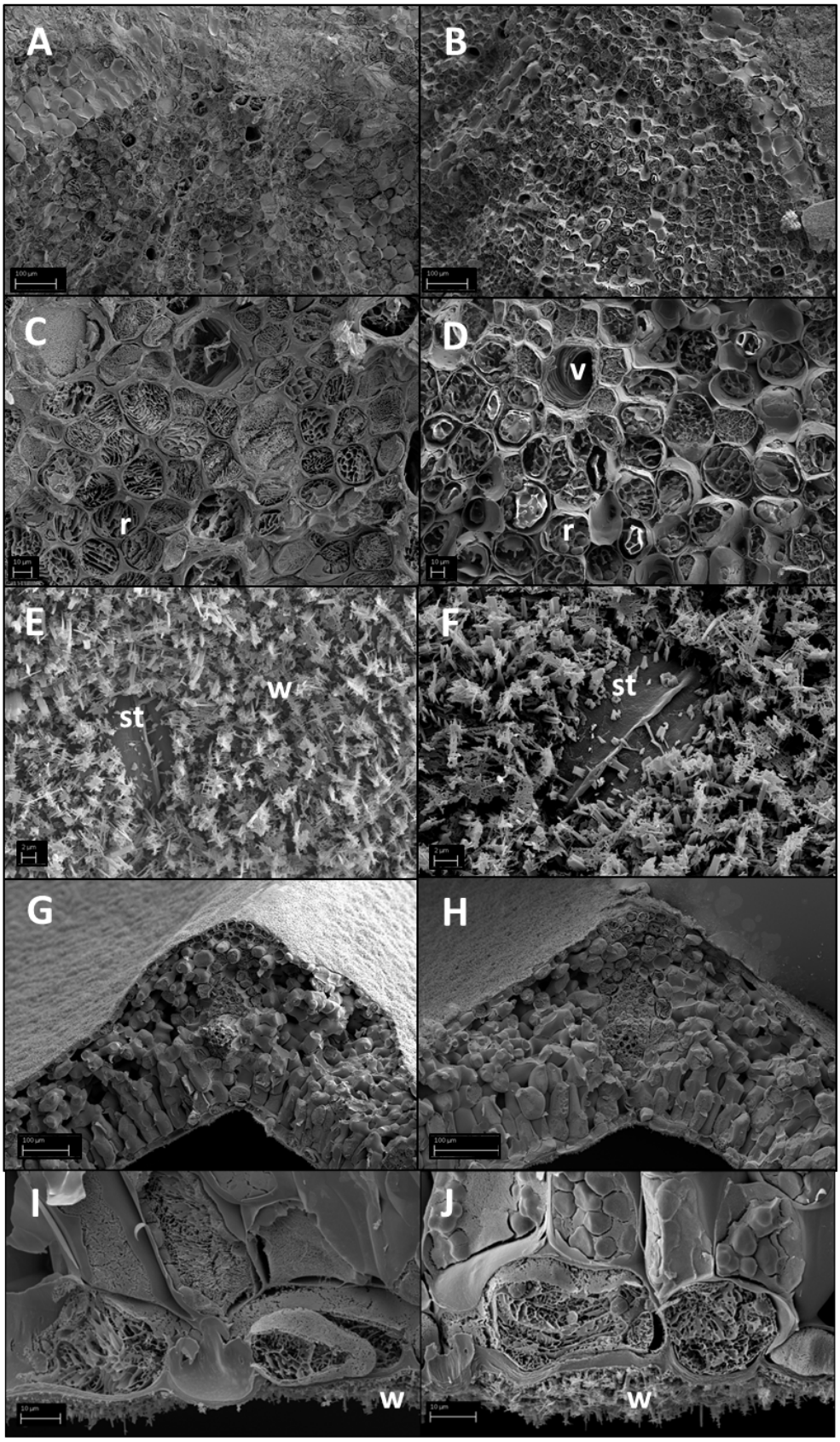


Figure 3.4.: Scanning Electron Microscopy images of *B. napus* var. Visby root (A - D) and leaf (E - J) tissue of control plants (left row) and 9 weeks after inoculation with *A. alternatum* (right row). No hyphae could be detected in roots (A - D), on the leaf surface (E, F) or in cross sections of leaves (G - J) in comparison to controls. 2 samples per treatment were examined. Abbreviations: r - root cell, v - vessel, st - stoma, w - cuticular wax. Scale bars represent 100 μm (A, B, G, H) and 2 μm (E, F).

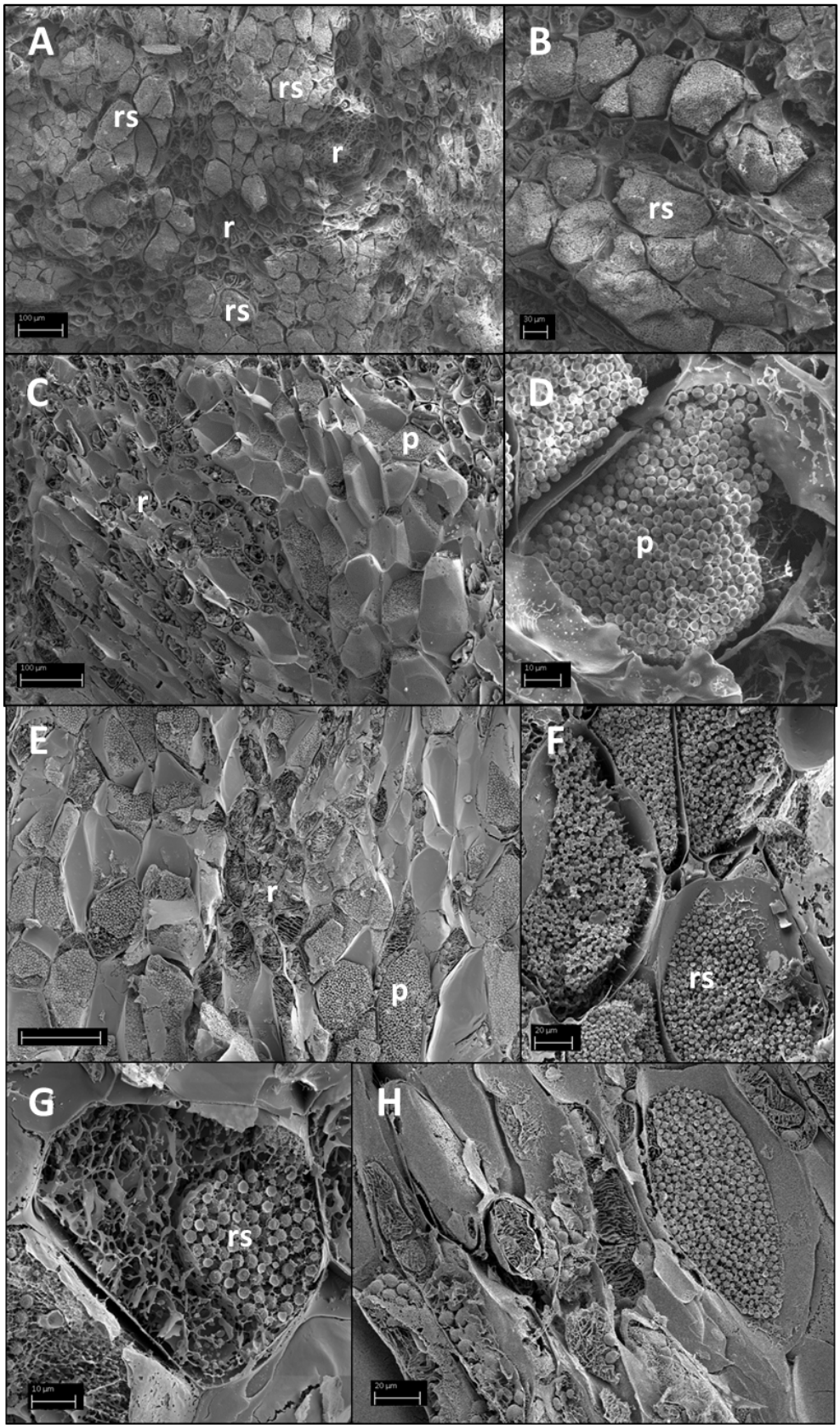


Figure 3.5.: Scanning Electron Microscopy images of *B. napus* var. Visby roots 9 weeks after inoculation with *A. alternatum* and *P. brassicae* (A - D) and with *P. brassicae* alone (E - H). 2 samples per treatment were examined. Abbreviations: r - root cells, v - vessel, st - stoma, w - cuticular wax. Scale bars represent 100 μm (A, B, G, H) and 2 μm (E, F).

3.1.3. Quantification of fungal and protist DNA in rapeseed tissues

To quantify *A. alternatum* and *P. brassicae* in rapeseed, a dilution series of genomic DNA of both organisms was prepared and qPCR was carried out. Each sample was run as quadruplet. These standard curves were used for the calculation of the DNA amount of both organisms in rapeseed. DNA was extracted from 2 tissue samples of rapeseed per treatment. The extraction out of hypocotyl did not yield much DNA which could be an effect of the high water content of this tissue. Consequently the amounts of DNA from this tissue were very low for both pathogens (Fig. **??**).

The amount of fungal DNA was relatively high in the single-treatment in comparison to plants that had been inoculated with both pathogens and also in relation to the amount of protist DNA in all samples inoculated with *P. brassicae* (Fig. **??**). Root samples inoculated with both organisms contained approximately the same amount of protist DNA than samples inoculated with *P. brassicae* alone (3,15 compared to 2,20 fg/mg fresh weight). However, the standard deviation of 2 samples per treatment was relatively high especially for the quantification of *A. alternatum* DNA. This was probably due to a different efficiency of the DNA extraction for the fungal DNA out of the plant tissue.

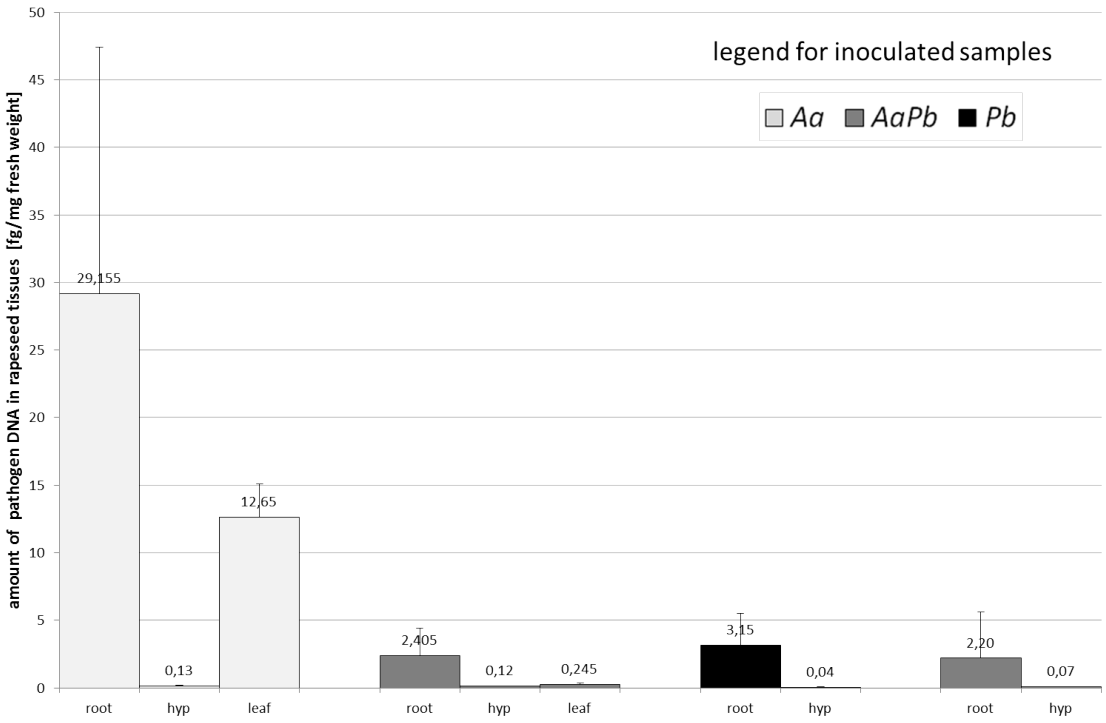


Figure 3.6.: Quantification of *P. brassicae* and *A. alternatum* DNA from inoculated rapeseed tissues. Total amount of DNA was calculation after qPCR with rapeseed tissue that contained the respective microorganism. Abbreviations: *Aa - A. alternatum, Pb - P. brassicae,* hyp - hypocotyl.

3.2. Disease rating of clubroot symptoms in Arabidopsis and Brassica

It was previously reported that a spore density of 10^7 spores/ml for *A. alternatum* is sufficient to achive a reduction of clubroot symptoms in Arabidopsis and Chinese cabbage (Jäschke et al. 2010; Doan et al. 2010). In this study 3 different spore densities were applied ranging from a low (10^5) over medium (10^6) to high concentration (10^7) to assess the effect of *A. alternatum* on clubroot development.

To test for the suceptibility of a plant against the clubroot disease, lower concentrations of disease inoculum were used whereas the tolerance of the plant for the pathogen was tested with higher concentrations of pathogen inoculum (see Table 3.2).

In Arabidopsis the co-inoculation with *A. alternatum* with a medium or high spore concentration resulted in a reduced disease index in comparison to clubroot infected plants which was significant in all experiments except 1 (Kruskal-Wallis test; p values were 0,008; 0,000; 0,453 for 10^6 and 0,000; 0,043 for 10^7spores/ml; Tab. 3.1). At the low spore concentration co-inoculated plants had a significantly higher DI than plants infected with clubroot alone (p=0,004; Kruskal-Wallis-test; Tab. 3.1).

In rapeseed the disease rating did not show a significant control of clubroot symptoms in rapeseed at any concentration of *A. alternatum* tested (p>0,05; Kruskal-Wallis test; Tab. 3.1; Auer and Ludwig-Müller (2014)).

Table 3.1.: Results of the disease rating of Arabidopsis and *B. napus* after inoculation with *A. alternatum* (+) and *P. brassicae* (-). The disease index (DI) refers to the DI average for all experiments with the respective spore concentration of both microorganisms.

Plant species	Spores per ml	Inoculation ± *A. alternatum*	n	DI (avg)	trials
Arabidopsis	10^5	+ / -	35 / 30	73 / 53	1
"	10^6	+ / -	118 / 115	82 / 87	3
"	10^7	+ / -	62 / 64	72 / 85	2
B. napus var. Ability	10^5	+ / -	16 / 16	80 / 83	1
B. napus var. Visby	10^6	+ / -	16 / 16	37 / 36	1
B. napus var. Ability	10^7	+ / -	14 / 14	62 / 76	1

3.3. Relative gene expression of resistance and MAMP-related genes

The main goal of this study was to find out whether the fungus *A. alternatum* induces resistance genes in Arabidopsis roots that lead to a reduction of clubroot symptoms in the roots as observed in Jäschke et al. (2010). For this, inoculation experiments were carried out and RNA from inoculated and control roots was extracted during different developmental stages of the clubroot pathogen within Arabidopsis.

The transcript changes upon pathogen and endophyte challenge were assessed by analysing a microarray from Arabidopsis roots conducted 3 days after treatment and with semi-quantitative and quantitative real time RT-PCR of resistance related genes at the cortical stage of infection with *P. brassicae*. The data from the microarray were partially verified with qRT-PCR of endophyte-inoculated Arabidopsis roots (see section 3.6.1.3).

In addition, root tissue of inoculated *B. napus* var. Visby was analysed with qRT-PCR for the regulation of 5 resistance genes where gene sequences and primers had been published previously (Wu et al. 2010; Wang et al. 2012).

To enhance the readability in the following sections, all inoculation experiments are abbreviated. Samples inoculated with *A. alternatum* **alone are abbreviated as "***Aa***", samples inoculated with** *A. alternatum* **and** *P. brassicae* **are "***AaPb***" and samples inoculated with** *P. brassicae* **alone are "***Pb***".**

3.3.1. Microarray results

An Agilent microarray (8x60K) was conducted on root tissue from *Arabidopsis* wildtype Col-0 from plants inoculated with *Pb* and *Aa*. The threshold for differential regulation of the raw data was set to $\geq$2,0 for upregulation and $\leq$0,5 for downregulation. In roots inoculated with *Aa* 207 genes, with *Pb* 152 and in the *AaPb* inoculation 270 genes were differentially regulated. The increased number of genes with altered transcript levels for the *AaPb* treatment was expected as the plant is challenged with 2 pathogens at once which leads to additive effects in terms of transcript levels.

For subsequent analysis all relative expression values were LOG2 transformed.

3.3.1.1. Overview of microarray results with Mapman

To visualise this complex dataset and get a better overview of the data genes with significantly altered transcript levels were analysed with Mapman (Thimm et al. 2004; see Fig. 3.7) and their function looked up in the The Arabidopsis Information Resource (TAIR) data

base (www.arabidopsis.org, last Date of access: May 2015). Mapman displays a graphic overview over large data sets and groups genes according to their function in pathways such as "Secondary metabolites" or "Biotic stress". Here, only the pathway "Biotic stress" will be discussed as the focus of this study was on genes related to defence mechanisms.

A complete list with all significant differentially expressed genes can be found in the appendix (Tab. A - C).

Auxin

The Mapman picture depicted that for the *Aa* treatment 3 auxin-related genes were up-regulated - *AT5G01100* that encodes for a GDP-fucose protein that transfers glycosyl groups and the 2 auxin-responsive genes *SAUR20* and *SAUR77 (SMALL AUXIN UPREGULATED RNA; AT1G17345* and *AT5G18020*) whose functions are yet unknown (Fig. 3.7). Some SAUR-family proteins can promote cell expansion presumably through modulation of auxin transport (Spartz et al. 2012).

In the *AaPb* samples *AT5G01100*, *SAUR71* (*AT1G56150*) and *GH3.1* (*AT2G14960*) were upregulated. *GH3.1* encodes a protein similar to the indole-3-acetic acid amido synthase and was also upregulated by 2-fold in a microarray with Arabidopsis roots inoculated with *Pb* from Siemens et al. (2006) at the early timepoint 10 dai and 1.2-fold at 23 dai. In the *Pb* samples of this study also *SAUR78* (*AT1G72430*) was upregulated.

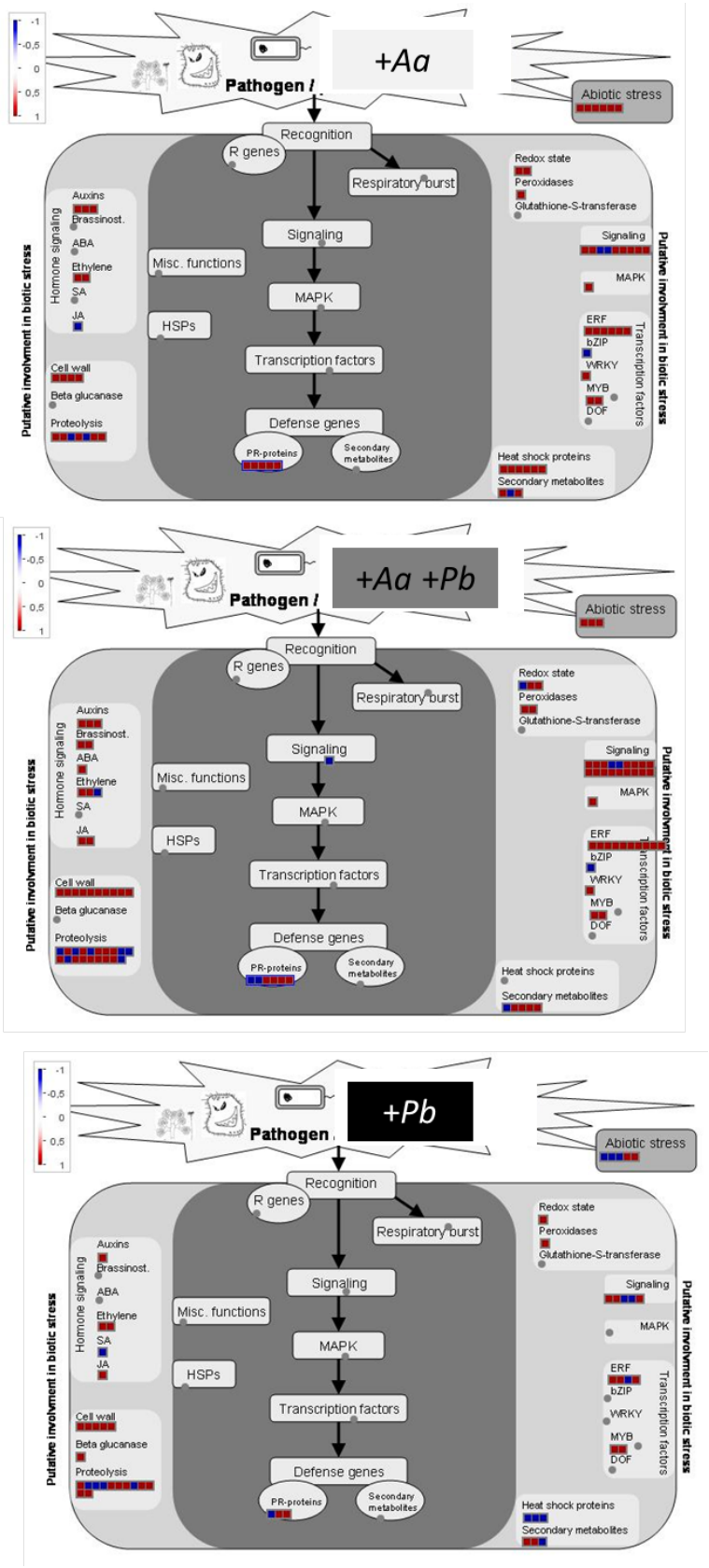


Figure 3.7.: Mapman analysis of microarray data from roots of Arabidopsis 3 days after inoculation with *A. alternatum* and *P. brassicae*. The picture shows the biotic stress pathway of genes susceptible to pathogen challenge. Colours indicate upregulation (red) and downregulation (blue) of the respective genes. Only genes with significantly differential transcript levels are displayed. The microarray was conducted on RNA extracted from Arabidopsis roots with a 8x60k Agilent array. Abbreviations: *Aa - A. alternatum, Pb - P. brassicae*

Brassinosteroids

In the *AaPb* samples 2 genes for brassinosteroids were upregulated: the *BRI1-ASSOCIATED RECEPTOR KINASE 1* (*BAK1*; *AT4G33430*), one of the MAMP-related-genes of this study (see section 3.3.1.2), and the *EXORDIUM-LIKE 5-GENE* (*EXL5*; *AT2G17230*) with yet unknown function.

ABA

One gene, *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*; *AT3G14440*) was significantly upregulated in the *AaPb* samples. *NCED3* encodes the 9-cis-epoxycarotenoid dioxygenase, a key enzyme in the biosynthesis of ABA.

Ethylene

Two members of the ET response family proteins (ERF) were upregulated in *Aa* roots: *ETHYLENE AND SALT INDUCIBLE 1* (*ESE1*; *AT3G23220*) and *ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 14* (*ERF14*; *AT1G04370*).

In the *AaPb* samples 2 *1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE* genes, *ACS7 and ACS8* (*AT4G26200*; *AT4G37770*), were upregulated which encode for enzymes involved in ET biosynthesis. The ET-responsive *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59* (*ORA59*; *AT1G06160*) was downregulated. In *Pb* roots 2 ET-related genes were upregulated: *AT5G12270* of which the protein has an oxidoreductase activity and *ACS11 (AT4G08040*).

Salicylic acid

For SA Mapman depicted only 1 gene as upregulated in the *Pb* samples, *URIDINE DIPHOSPHATE GLYCOSYLTRANSFERASE 74E2* (*UGT74E2*; *AT1G05680*), a UDP-glucosyltransferase that acts on indole-3-butyric acid and affects auxin homeostasis.

Jasmonate

In *Aa* roots Mapman displayed the downregulation of the JA-responsive gene *LIPOXYGENASE 2* (*LOX2*; *AT3G45140*). As a key enzyme in the octadecanoid-pathway *LOX2* is required for the pathogen-induced formation of JA and LOX2-levels directly correlate with the amount of JA produced (Spoel et al. 2003). Another *LOX*-gene, *LOX4* (*AT1G72520*), was induced in *AaPb* and *Pb* roots. The *EPITHIOSPECIFIER PROTEIN*-gene (*ESP*; *AT1G54040*), which encodes for a protein that interacts with WRKY53 in pathogen interactions, was upregulated specifically in *AaPb* roots.

Pathogenesis-related (PR) - proteins

Five genes were upregulated upon inoculation with *Aa*: *PLANT DEFENSIN 1.2 (PDF1.2*; see section 3.3.1.2), 2 genes encoding for leucine-rich repeat (LRR) family proteins; *AT5-G66890* and *AT5G41750*, as well as 2 genes known to be involved in the defence response to fungi; *LOW-MOLECULAR-WEIGHT CYSTEINE-RICH 25* (*LCR25*; *AT4G29305*) and *AT3G24510*, which encodes a defensin like family protein (DEFL).

For *AaPb* 4 *PR*-genes were upregulated: 3 genes encoding for disease resistance proteins of the TIR-NBS-LRR class (*AT1G57630*, *AT5G41750*, *AT1G72950*) (toll-interleukin-resistance-nucleotide binding site-LRR) and 1 DEFL-protein (*AT1G11055*), whereas 2 *PR*-genes were downregulated: *RESISTANCE TO LEPTOSPHAERIA MACULANS 3* (*RLM3*; *AT4G16990*), which might be a R-protein specific downstream signaling component for a resistance pathway to necrotrophic fungi (Staal et al. 2008) and *AT1G50180* which encodes for a protein of the CC-NBS-LRR class and was also downregulated in *Pb* samples.

Specific for the *Pb* samples was the upregulation of *AT1G73325* encoding for a protease inhibitor and *AT5G52145* which is supposed to be a pseudogene of a DEFL-family protein. Here, *AT1G50180* was downregulated as well.

WRKY and MYB transcription factors

In *Aa* samples *WRKY DNA-BINDING PROTEIN 18* (*WRKY18*; see section 3.3.1.2) was upregulated. In *AaPb* samples *WRKY46* (*AT2G46400*) was upregulated.

MYB DOMAIN CONTAINING PROTEIN 79 (*MYB79*; *AT4G13480*) was upregulated in *Aa* samples, *MYB77* (*At3G50060*) in *AaPb* and *MYB22* (*AT5G40430*) in *Pb* samples. *MYB95* (*AT1G74430*) was upregulated in all treatments. According to TAIR, *MYB95* responds to JA as well as to SA and is therefore a rather unspecific *MYB* gene.

Other genes

The Mapman pathway for biotic stress also included other categories such as "proteolysis" or "heat shock proteins" which are not directly involved in resistance pathways and are therefore not discussed here. In the category "signaling" mainly genes for calcium-binding proteins and calmodulin-related proteins were upregulated. The *MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 18* (*MAPKKK18*; *AT1G05100*) was upregulated in *Aa* and *AaPb* samples.

Notable was the amount of cell wall related genes, which was highest in the *AaPb* inoculated samples (Fig. 3.7). However, cell wall synthesis and reconstruction are complex mechanisms with hundreds of genes involved. In this array no notable regulation of key enzymes for cell wall reconstruction were found.

3.3.1.2. Regulation of MAMP-associated and resistance genes

Most MAMP-related genes showed a relatively high response and 2 of the 7 genes investigated in this study were significantly induced - *BAK1* in *AaPb* and *Pb* treatments and *WRKY18* in *Aa* samples (see Fig. 3.8).

BAK1 was induced in all treatments with the strongest response to the *Pb* inoculation (Fig. 3.8). BAK1 is LRR-receptor kinase required by most known pattern recognition receptors for their function and therefore a central regulator of PAMP-triggered immunity (PTI) (Dodds and Rathjen 2010). The gene of FLAGELLIN SENSING 2 (FLS2), a receptor kinase in the membrane that can perceive the bacterial MAMP flg22 and forms an active complex with BAK1, was slightly induced upon co-inoculation with *Aa.* Interestingly, *FLS2* showed lower transcript levels in the *Pb* samples of the microarray which suggests that no interaction of FLS2 with BAK1 was possible at 3 dai thus effectively blocking PTI whereas in the *AaPb* samples an interaction of both proteins seems likely.

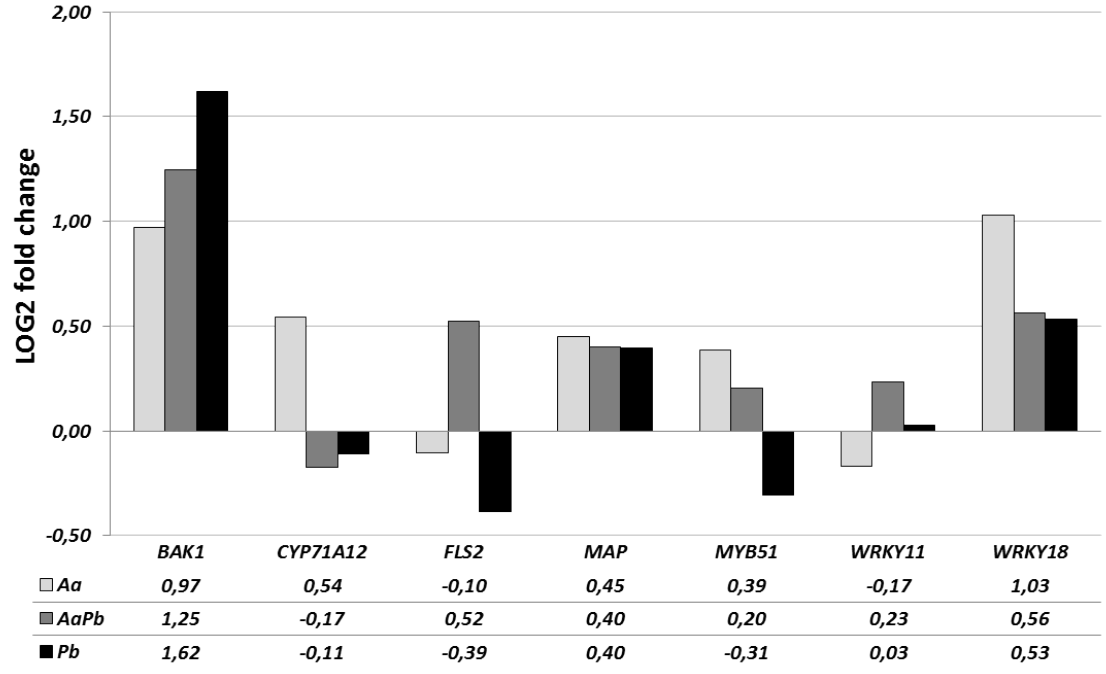
The cytochrom P450 gene *CYP71A12* was slightly activated upon *Aa* inoculation (LOG2 fold change 0,54). CYP71A12 is involved in the camalexin biosynthesis and the gene was upregulated in *Pb*-Arabidopsis roots in the microarray from Siemens et al. (2006) at 23 dai. *MAP*, a nodulin-like gene with yet unknown function, was slightly induced in all treatments.

MYB51 was induced upon *Aa* and *AaPb* treatment and downregulated upon the *Pb* inoculation (Fig. 3.8). *WRKY11* was slightly activated upon *AaPb* inoculation only. WRKY11 responds to flg22 and is a negative regulator of basal resistance in Arabidopsis (Millet et al. 2010).

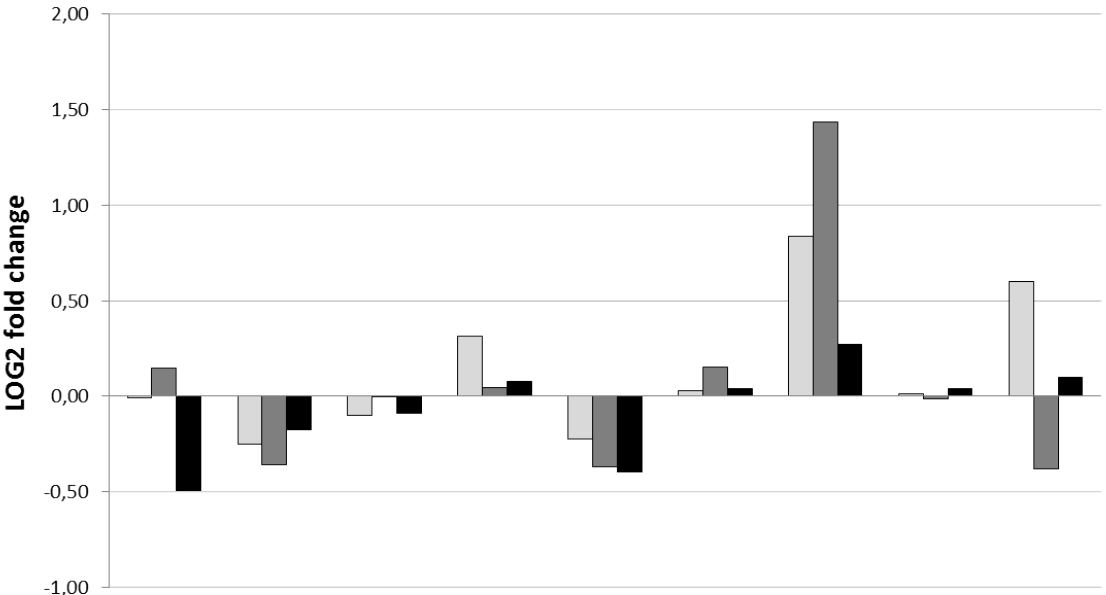
WRKY18 was strongly induced upon *Aa* inoculation and to a lesser extent after *AaPb* and *Pb* treatment. WRKY18 is a positive transcription regulator that is required for the full induction of the systemic aquired resistance (SAR) (Wang et al. 2006).

Generally the response amplitude of the resistance genes was very low and did not reach the threshold level of ±1 on the logarithmic scale, except for *PDF1.2* which was induced at 3 dai in *AaPb* samples (3.8). This was expected, as all of these genes act downstream of the MAMP-related genes and are induced at later time points as described in the next section.

NPR1 is mainly post-transcriptionally regulated and did not show a regulation as well as *ETR1,* the ethylene receptor and the isochorismate synthase gene *SID2*. The levels of *PR1* in *Pb* samples were lower than the controls and no regulation was observed in the *Aa* or *AaPb* samples at this timepoint. *GH3.5* was slightly activated in *Aa* samples and not regulated in all other treatments (3.8).



	BAK1	CYP71A12	FLS2	MAP	MYB51	WRKY11	WRKY18
Aa	0,97	0,54	-0,10	0,45	0,39	-0,17	1,03
AaPb	1,25	-0,17	0,52	0,40	0,20	0,23	0,56
Pb	1,62	-0,11	-0,39	0,40	-0,31	0,03	0,53



	PR1/CAP	COI1	ETR1	GH3-5	JAR1	NPR1	PDF1.2	SID2	THI2.1
Aa	-0,01	-0,25	-0,10	0,31	-0,22	0,03	0,84	0,01	0,60
AaPb	0,15	-0,36	-0,01	0,04	-0,37	0,15	1,44	-0,02	-0,38
Pb	-0,49	-0,18	-0,09	0,08	-0,40	0,04	0,27	0,04	0,10

Figure 3.8.: Microarray data for MAMP-responsive genes and resistance genes. Data are obtained from a microarray with roots of Arabidopsis 3 days after inoculation with *A. alternatum* (*Aa*) and *P. brassicae* (*Pb*). The LOG2 fold change is relative to the control samples.

3.3.1.3. Conclusion of microarray data: A. alternatum primes clubroot infected Arabidopsis

The microarray data from root samples 3 dai showed a significant upregulation of several MAMP-responsive genes upon *Aa* inoculation among them *WRKY18*, a transcription

factor crucial for the biosynthesis of SA. Generally co-inoculation with the endophyte resulted in an increased amplitude of response for some defence related genes, indicating that the fungus primes Arabidopsis roots against clubroot. Priming is defined as a mild induction of MAMP-related genes upon recognition of beneficial microbes which lead to an enhanced resistance response upon subsequent pathogen attack.

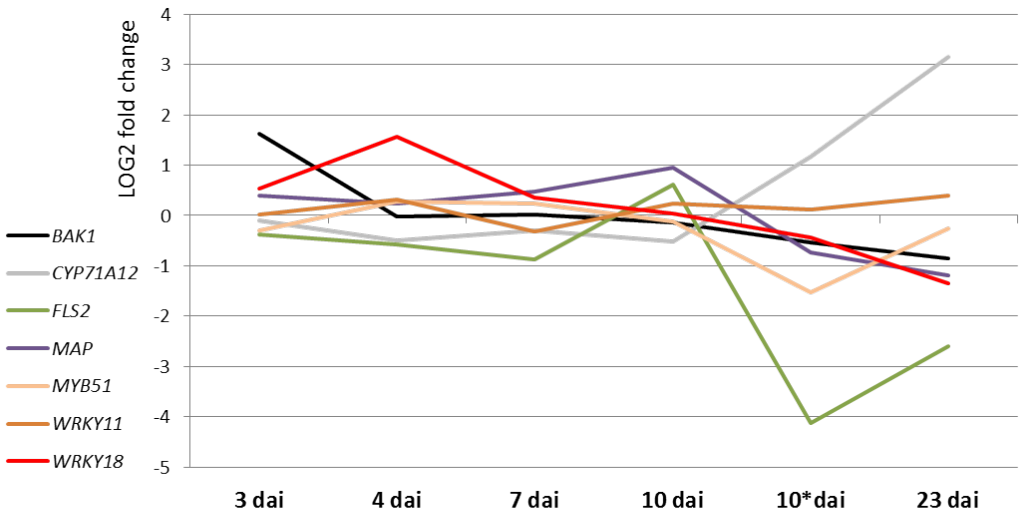
Generally, the data from this microarray suggest that upon recognition of the clubroot pathogen several defence mechanisms are suppressed: *FLS2, JAR1* and *PR1* were slightly downregulated thus likely impairing the PAMP-triggered immunity (PTI) response of the plant as well as an early onset of systemic acquired resistance (SAR). Furthermore the slight deactivation of *MYB51* presumably affects glucosinolate biosynthesis negatively (Fig. 3.8).

Co-inoculation with *Aa* led to higher transcript levels of *WRKY18*, *WRKY11*, *MYB51*, *FLS2*, *PDF1.2* and *PR1* in the *AaPb* samples and likely reversed negative effects of the clubroot pathogen by enabling PTI, SAR and glucosinolate biosynthesis. *BAK1* was weaker induced in *AaPb* samples than in *Pb* samples. This suggests that a lower level of *BAK1* might be sufficient to establish PTI.

Inoculation with Aa induced *PDF1.2* and *THI2.1* and points to an activated JA-related defence mode. However, this was not supported by the regulation of other JA-responsive genes - *LOX2*, *COI1* and *JAR1* were either downregulated or did not show any response.

3.3.1.4. Comparison with other Arabidopsis root microarrays

2 Affymetrix microarrays of Arabidopsis Col-0 roots inoculated with *Pb* are available from the literature. Agarwal et al. (2011) used an Australian spore isolate from the field and analysed 3 early time points: 4, 7 and 10 dai. Siemens et al. (2006) used the same single spore isolate e3 that was utilized in this study and analysed roots from 10 dai and 23 dai. Agarwal et al. (2011) found a strong upregulation of *WRKY18* at 4 dai followed by a constant decline in transcript levels until 10 dai (see 3.9). In the second array *WRKY18* was slightly reduced at 10 dai and downregulated at 23 dai (Siemens et al. 2006; Fig. 3.9).



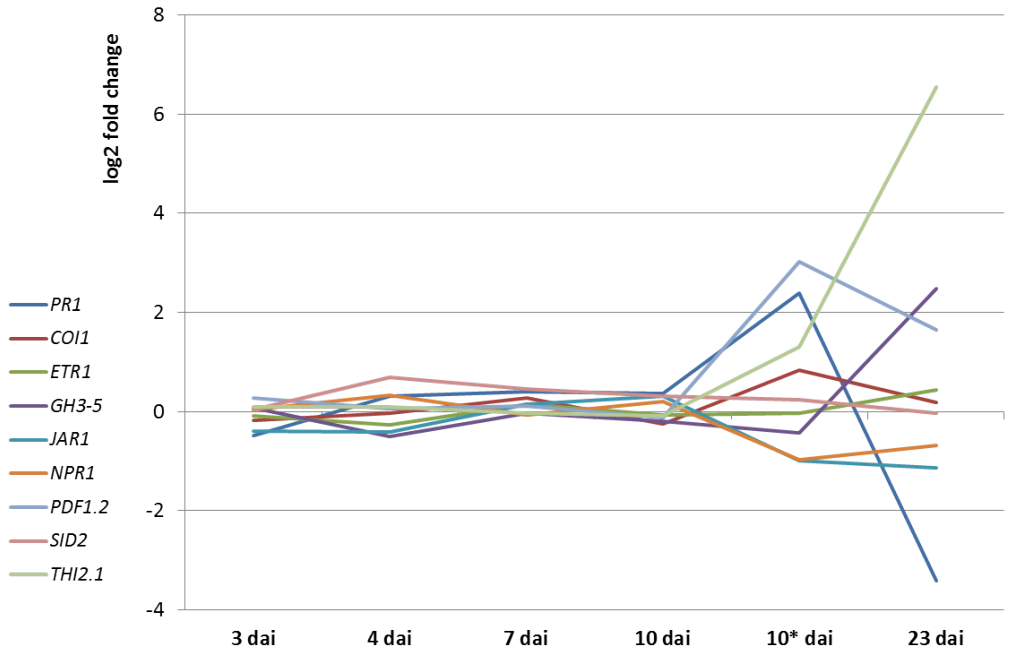
	3 dai	4 dai	7 dai	10 dai	10*dai	23 dai
BAK1	1,62	-0,01	0,01	-0,13	-0,54	-0,85
CYP71A12	-0,11	-0,49	-0,30	-0,51	1,16	3,16
FLS2	-0,39	-0,57	-0,88	0,61	-4,13	-2,59
MAP	0,40	0,24	0,47	0,95	-0,74	-1,19
MYB51	-0,31	0,28	0,24	-0,11	-1,53	-0,26
WRKY11	0,03	0,32	-0,31	0,24	0,12	0,40
WRKY18	0,53	1,56	0,36	0,03	-0,43	-1,36

Figure 3.9.: Expression profiles of microbe-associated molecular pattern–related genes in Arabidopsis roots after inoculation with *P. brassicae*. The values originate from 2 published microarrays from Agarwal et al. (2011) (4, 7 and 10 days), Siemens et al. (2009) (10* and 23 days) and an unpublished microarray from this thesis (3 dai). Colours in the data table depict the strength of the response; red indicates upregulation, blue downregulation. Abbreviations: dai - days after inoculation.

BAK1 was not upregulated at any other timepoint in the other arrays, in fact it was slightly downregulated starting from 10 dai and declining further until 23 dai. *FLS2* was downregulated at all timepoints with the lowest level at 10 dai in the array from 2006, in contrast to the 2010 array where a slight induction was observed at 10 dai with the Australian isolate (Fig. 3.9).

For *CYP71A12* an increase in transcript levels until 23 dai can be assumed according to all 3 arrays. As earlier mentioned, this protein is involved in the biosynthesis of the phytoalexin camalexin, which is typically produced upon infection with necrotrophic fungi (Nafisi et al. 2007). Transcript levels of *MAP*, *MYB51* and *WRKY11* did not show a clear trend over the 5 timepoints.

An overview of the resistance genes investigated at later time time points can be found in Fig. 3.10. All genes showed rather subtle changes in transcript levels until 10 dai. The expression profile of all genes over the whole time period clearly shows that these genes play a role in later stages in the clubroot development.



	3 dai	4 dai	7 dai	10 dai	10* dai	23 dai
PR1	-0,49	0,30	0,40	0,36	2,40	-3,41
COI1	-0,18	-0,03	0,27	-0,26	0,84	0,19
ETR1	-0,09	-0,27	0,14	-0,06	-0,03	0,43
GH3-5	0,08	-0,50	-0,03	-0,20	-0,44	2,47
JAR1	-0,40	-0,41	0,15	0,30	-0,99	-1,14
NPR1	0,04	0,32	-0,06	0,21	-0,97	-0,68
PDF1.2	0,27	0,06	0,11	-0,12	3,02	1,65
SID2	0,04	0,68	0,45	0,31	0,24	-0,04
THI2.1	0,10	0,09	-0,04	-0,08	1,30	6,55

Figure 3.10.: Expression profiles of resistance genes in Arabidopsis roots after inoculation with *P. brassicae*. The values originate from 2 published microarrays from Agarwal et al. (2011) (4, 7 and 10 days), Siemens et al. (2009) (10* and 23 days) and an unpublished microarray from this thesis (3 dai). Colours in the data table depict the strength of the response; red indicates upregulation, blue downregulation. Abbreviations: dai - days after inoculation.

3.3.2. Quantitative PCR results for Arabidopsis

Arabidopsis Col-0 were inoculated with *A. alternatum* and *P. brassicae* and roots harvested at 9, 14 and 20 dai. RNA was extracted from the roots and transcribed to cDNA to perform PCR. This experiment was done on soil and sand (=hydroponic culture) and repeated once for each substrate. The soil experiments were analysed with semi-quantitative (sq)RT-PCR and the hydroponic cultures with quantitative (q)RT-PCR.

For the sqRT-PCRs the cDNA samples were diluted and PCR was performed with the reference gene *YLS8* until all bands of one experiment showed the same intensity on an agarose gel. After this, PCR was performed with the primers for all genes of interest (GOI) and the intensity of the bands were compared on agarose gels.

For qRT-PCR a working concentration of 1:100 of the cDNA was used in all PCRs if not indicated otherwise. From the Ct values obtained in the PCRs relative gene expression was calculated by the $\Delta\Delta$Ct method (Livak and Schmittgen 2001). From the 2 independ-

ent experiments conducted on soil with Arabidopsis only 1 yielded enough RNA to carry out all necessary PCR analyses.

For none of the 2 experiments conducted on sand a complete dataset for the timepoint 20 dai could be achieved due to difficulties to obtain enough RNA for all treatments. In consequence, only a smaller set of relative expression data is available for the timepoint 20 dai for the qPCR experiments.

Additionally, primers for *SID2* stopped to work properly at some time point during the qPCR experiments maybe due to contamination and yielded unspecific products. Because of time restrictions the PCR experiment was not repeated and only data from 1 timepoint is available for *SID2*.

The expression of *THI2.1* and *PDF1.2* should be interpreted with caution as this genes are expressed only in small amounts in roots and a much higher concentration of cDNA was needed to amplify specific gen products for these 2 genes. Normally, a 1:100 dilution of each cDNA was used in qPCR for genes of interest and reference genes. For analysis of *PDF1.2* and *THI2.1* a cDNA dilution of 1:10 or 1:25 was used for both genes of interest and the respective reference genes to achieve a signal in the qPCR experiments. For sqRT-PCR 38 to 40 cycles were used to be able to detect a signal of the amplificate on the gel.

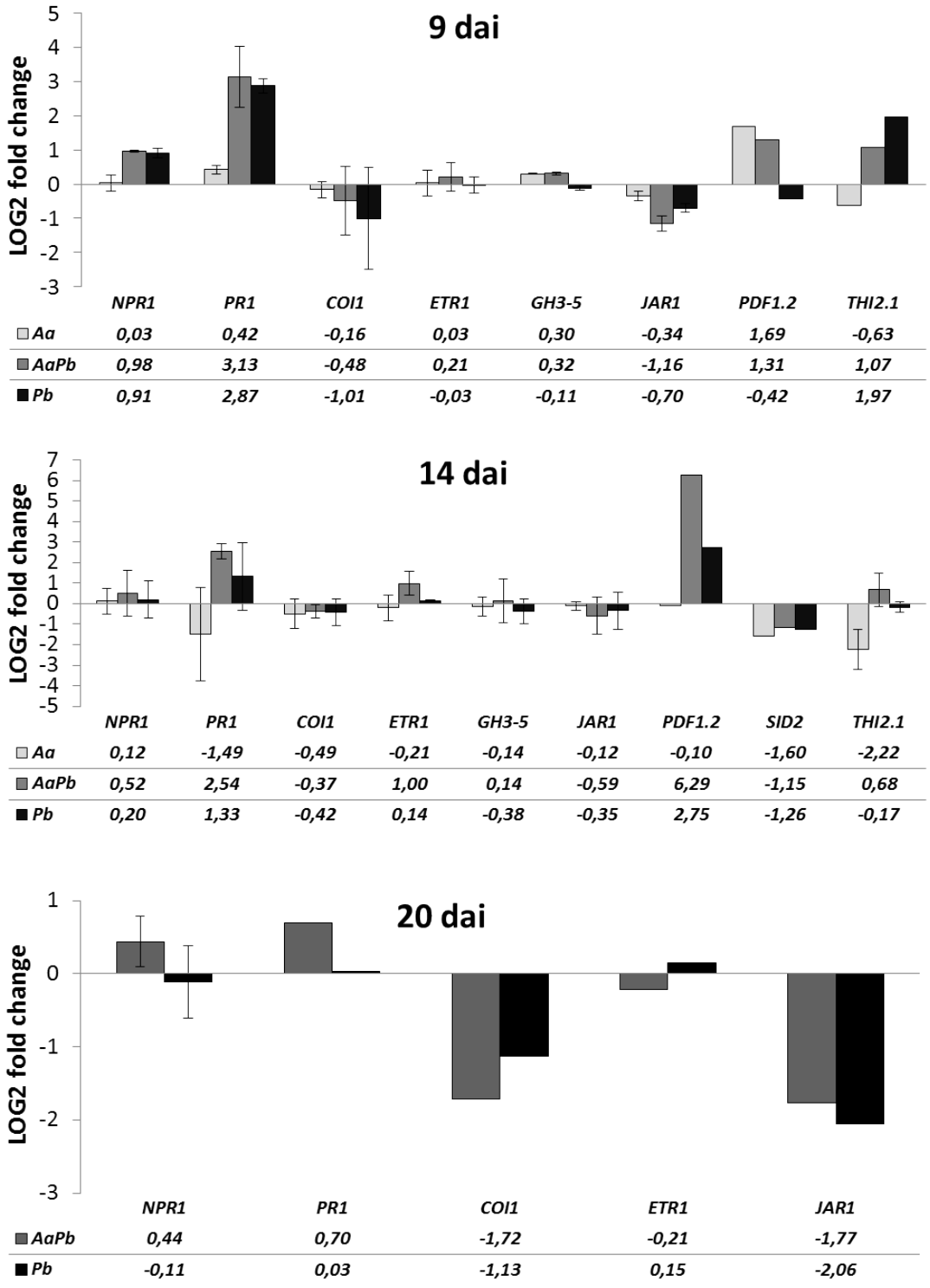
3.3.2.1. Relative expression of resistance genes from hydroponic cultures (qRT-PCR)

Of the 9 resistance genes investigated 5 showed significantly altered transcript levels upon treatment with *Aa* and *Pb* during the stage of cortical infection: *PR1*, *COI1*, *JAR1*, *PDF1.2* and *THI2.1*.

NPR1 and *GH3.5* did not show considerable changes in their expression. *ETR1* was not regulated except for *AaPb* samples at 14 dai where an upregulation was found (LOG2 fold change 1,00) (Fig. 3.11).

PR1 was strongly induced in the *AaPb* samples (LOG2 fold change 3,13 at 9dai, 2,54 at 14 dai) and to a lesser extent in the *Pb* samples at 9 dai and 14 dai (LOG2 fold change 2,87; 1,33; Fig. 3.11). At 20 dai *PR1* still showed a weak signal (LOG2 fold change 0,70) in *AaPb* samples whereas in *Pb* roots no regulation was visible at this timepoint in the qPCR experiments (0,03). As *PR1* is a marker for successful SAR these results indicate that the endophyte helped establish and maintain SAR in the *AaPb* samples throughout the whole secondary infection with clubroot in Arabidopsis roots. In *Aa* samples *PR1* was repressed at 14 dai (LOG2 fold change -1,49).

Data for *SID2* were only available for 14 dai as explained in the previous section and for the sqRT-PCR experiments. As with *COI1* and *JAR1* a downregulation was observed for *SID2* with similar levels for all treatments (LOG2 fold change for *Aa*: -1,60; *AaPb*: -1,15; *Pb*: -1,26).



	NPR1	PR1	COI1	ETR1	GH3-5	JAR1	PDF1.2	THI2.1
Aa	0,03	0,42	-0,16	0,03	0,30	-0,34	1,69	-0,63
AaPb	0,98	3,13	-0,48	0,21	0,32	-1,16	1,31	1,07
Pb	0,91	2,87	-1,01	-0,03	-0,11	-0,70	-0,42	1,97

	NPR1	PR1	COI1	ETR1	GH3-5	JAR1	PDF1.2	SID2	THI2.1
Aa	0,12	-1,49	-0,49	-0,21	-0,14	-0,12	-0,10	-1,60	-2,22
AaPb	0,52	2,54	-0,37	1,00	0,14	-0,59	6,29	-1,15	0,68
Pb	0,20	1,33	-0,42	0,14	-0,38	-0,35	2,75	-1,26	-0,17

	NPR1	PR1	COI1	ETR1	JAR1
AaPb	0,44	0,70	-1,72	-0,21	-1,77
Pb	-0,11	0,03	-1,13	0,15	-2,06

Figure 3.11.: Gene expression data from qRT-PCR of Arabidopsis roots inoculated with *A. alternatum* and *P. brassicae* (10^7 spores/ml). All data are LOG2-transformed, the bars show the fold change of gene expression relative to controls. Where the standard deviation is missing only 1 PCR was performed. Abbreviations: dai - days after inoculation, *Aa* - *A. alternatum*, *Pb* - *P. brassicae*.

COI1 was downregulated in *Pb* samples at 9 dai (-1,01) and to a lesser extent in *AaPb*

(-0,48) at this timepoint whereas the opposite was observed at 20 dai - here the downregulation was stronger in the *AaPb* samples (-1,72) than in *Pb* roots (-1,13).

JAR1 was downregulated as well in most treatments: the strongest repression was observed at 20 dai in *Pb* samples (-2,06) and in *AaPb* samples at 20 dai (-1,77) and 9 dai (-1,16; Fig. 3.11).

ETR1 was induced only in the *AaPb* samples at 14 dai (1,00) and not regulated in the other treatments and timepoints (Fig. 3.11).

PDF1.2, another marker for JA, was upregulated in *Aa* samples early (9dai; 1,69) and not regulated later (0,10), in *AaPb* samples the gene was induced early (9dai; 1,31) and even stronger later (14 dai; 6,29). In *Pb* samples *PDF1.2* showed a slight deactivation at 9 dai (-0,42) and an induction 14 dai (2,75). The expression of *THI2.1* was the opposite of the *PDF1.2* expression at 9 dai: slight deactivation in *Aa* (-0,63), induction in *AaPb* (1,07) and stronger induction in *Pb* samples (1,97). At 14 dai *THI2.1* was strongly downregulated in *Aa* (-2,22), slightly activated in *AaPb* (0,68) and not regulated in *Pb* samples (-0,17). An upregulation of 1 or both of these genes suggests activation of the JA/ET-mediated defence pathways. However, this was not supported by the data for *COI1*, *JAR1* and *ETR1*.

3.3.2.2. Relative expression of resistance genes from soil grown Arabidopsis (sqRT-PCR)

Some of the findings from the qPCR experiments were also found in the soil experiment. Here, the same 5 genes appeared to be differentially regulated.

NPR1 showed a strong signal in the control at 9 dai and no clear regulation in the other samples. It is likely that this was rather an artefact of the cDNA dilution than a real induction and no induction could be observed at any later timepoint. For *GH3.5* and *ETR1* no regulation could be observed.

The induction of *PR1* in *AaPb* samples was strong and reproducible, the highest levels were found at 20 dai (Fig. 3.12). Here, *PR1* was induced in the *Aa* and *Pb* samples as well though to a lesser extent than in the *AaPb* sample. At 9 dai the gene was downregulated in *Pb* inoculated roots.

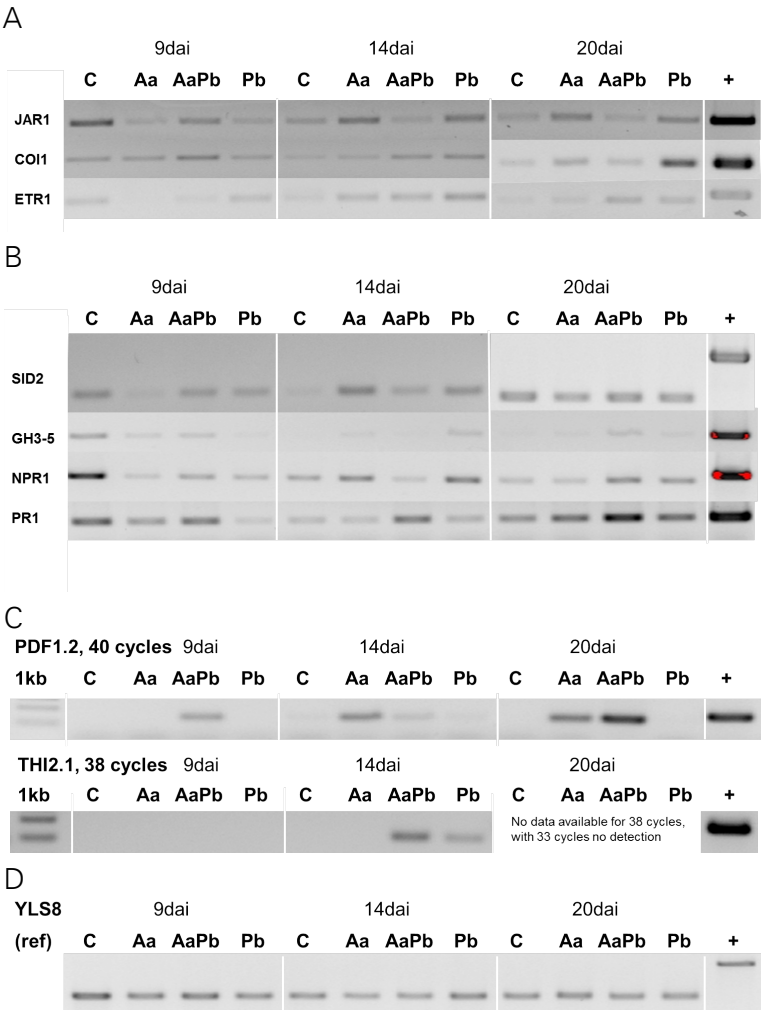


Figure 3.12.: Semi-quantitative RT-PCR results from Arabidopsis roots grown on soil. Pictures show representative gel slides after PCR with cDNA from Arabidopsis roots, treated with *A. alternatum* and *P. brassicae*. To obtain cDNA 1 µg root RNA was transcribed with random hexamer primers and cDNA was diluted 1:10 to obtain a stock solution. The stock cDNA was then normalized (further diluted) using the reference gene *YLS8* (see last row D), for each gene 30 to 34 PCR cycles were performed, except when indicated otherwise. Intensity of bands indicates strong expression of the particular gene. The figure shows the expression level of genes involved in the salicylic acid mediated pathway (A) and jasmonate and ethylene mediated pathway (B and C). Abbreviations: dai = dai, C = control, Aa = inoculated with *A. alternatum*, AaPb = mixed inoculation with *A. alternatum* and *P. brassicae*, Pb = inoculated with *P. brassicae*, + indicates positive control with genomic DNA from Arabidopsis seedlings.

The expression pattern for *SID2* varied from PCR to PCR so that no clear conclusion could be drawn. *COI1* seemed to be induced at 9 dai in *AaPb* samples and probably in *Pb* samples at 14 and 20 dai as well. The signal at 20 dai was strong but not reproducible in all PCRs. *JAR1* seemed to be induced in *AaPb* samples at 9 dai but not so at the other timepoints. In contrast, strong signals were found in *Aa* and *Pb* treatments at 14 and 20 dai.

The most interesting results were obtained with *PDF1.2* and *THI2.1:* According to the gel pictures, *PDF1.2* seemed to be specific for the inoculation with *Aa* as a signal could

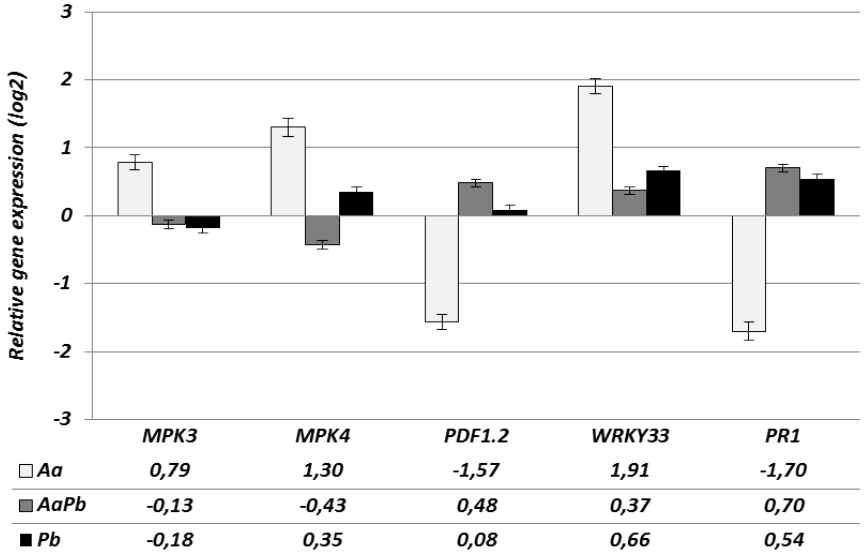
only be detected in samples with the endophyte. A high cycle number (40) was necessary to be able to detect a signal at all. *THI2.1* was equally hard to amplify and it took 38 cycles to see a band at samples inoculated with *Pb* and *AaPb*. These resulst suggested that *PDF1.2* could be a marker specific for the inoculation with the endophyte whereas *THI2.1* might serve as marker gene for the clubroot infection.

Overall, the upregulation of *PR1* in *AaPb* samples was highly reproducible and could be confirmed with this method. The results of all other genes were not so clear, except for the potential use of *PDF1.2* and *THI2.1* as specific markers for the fungal or clubroot infection respectively.

3.3.3. qRT-PCR results for Brassica

B. napus var. Visby plants from hydroponic cultures were inoculated with *A. alternatum* and *P. brassicae* and RNA was extracted as described in section 3.3.2. The timepoint 3 dai was chosen for the investigation of defence genes and the experiment was repeated once with similar results (data not shown).

The single treatment with *A. alternatum* induced the map kinase gene *BnMAPK4* and the transcription factor *BnWRKY33* and repressed *BnPR1* and *BnPDF1.2* (Fig. 3.13). No induction or repression was observed in samples inoculated with *P. brassicae* or co-inoculated with both organisms that met the threshold criteria ($\geq$1 or $\leq$-1 on a logarithmic scale) (Fig. 3.13).



	MPK3	MPK4	PDF1.2	WRKY33	PR1
Aa	0,79	1,30	-1,57	1,91	-1,70
AaPb	-0,13	-0,43	0,48	0,37	0,70
Pb	-0,18	0,35	0,08	0,66	0,54

Figure 3.13.: Relative gene expression of MAMP-related genes and *PR1* in *B. napus* var. Visby roots 3 days after inoculation with *A. alternatum* (*Aa*) and *P. brassicae* (*Pb*) (10^7spores/ml). Shown are the results from 1 experiment, another experiment yielded similar results (data not shown). Error bars are standard deviations of 2 PCRs.

3.4. Mutant analyses to verify qPCR and microarray data

To verify some of the microarray and PCR results Arabidopsis mutants were used for functional analyses.

The transcription factor *WRKY18* is a positive transcriptional regulator required for the full induction of SAR and plays a crucial role in the interaction of *P. brassicae* with the host plant Arabidopsis (Wang et al. 2006; Agarwal et al. 2011). In this study Arabidopsis plants treated with *A. alternatum* showed an early upregulation of *WRKY18* at 3 dai thus indicating that the endophyte probably induces SA-mediated defence responses at the beginning of the interation.

To further assess the role of *WRKY18 in vivo* the T-DNA mutant *wrky18* (Alonso et al. 2003) and 2 overexpressor lines, *wrky18oe1* and *wrky18oe2* (Chen and Chen 2002; Potschin et al. 2014), were inoculated with *A. alternatum* and *P. brassicae*.

At a low concentration of inoculum (10^5 spores/ml of *A. alternatum* and *P. brassicae*) clubroot infected *wrky18* were slightly more susceptible to the pathogen than the wild-type Col-0 and slightly less susceptible when co-inoculated with *A. alternatum* (Fig. 3.2). For Col-0 the difference between the treatment groups was significant (p=0,004; Kruskal-Wallis-test). It is notable, that the biomass of co-inoculated *wrky18* was reduced by 45% in comparison to the control plants while the other treatment groups displayed the same biomass as the control group. In the wildtype the biomass of co-inoculated and clubroot-inoculated plants was 46 or 47% higher than the controls and in the endophyte treated plants a biomass increase of 25% was observed.

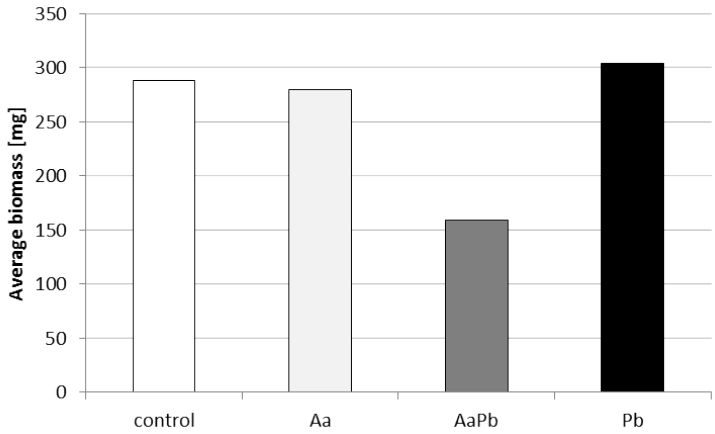


Figure 3.14.: Biomass of *wrky18* mutants 4 weeks after inoculation with *A. alternatum* (Aa) and *P. brassicae* (Pb) at the time point of the disease rating. Displayed is the average biomass of plants from 1 experiment. Spore concentration used was 10^5spores/ml.

At the higher concentration, the mutant was slightly less susceptible to clubroot than the wildtype in both treatments and the differences between both treatments was significant (p=0,007; Kruskal-Wallis-test; Fig. 3.2). Co-inoculated *wrky18* had a higher DI

than the clubroot infected plants and the difference was more prominent at this spore concentration. The biomass was not recorded in this experiment.

The overexpressor line 2 for WRKY18 was significantly less susceptible at the high spore concentration than the wildtype with a DI of 74 that can be considered as moderately resistant since it it close to the cut-off value of 70 for tolerance/moderate resistance (co-inoculated vs. clubroot-only: DI 74 vs. 83 in Col-0; p=0,005 for combined inoculation, DI 80 vs. 90 for Col-0, p=0,023 for clubroot inoculation; Kruskal-Wallis-test), at the lower spore concentration (10^6) no differences were observed regarding the DI for the overexpressors.

Overexpression of *WRKY18* led to moderately resistant plants in the combined inoculation with *A. alternatum* suggesting that the fungus boosts SA-related defence mechanisms of the plant against the clubroot disease.

In the microarray from this study the *BRI1-ASSOCIATED RECEPTOR KINASE 1*-gene *BAK1* was upregulatd by *P. brassicae* as well as in in the central cyclinder of Arabidopsis roots at 14 dai suggesting a role of brassinosteroids in the interaction of Arabidopsis with *P. brassicae* (Schuller et al. 2014). The authors reported that the receptor mutant *bri1-6* was less susceptible to clubroot than the wildtype. In this study the clubroot-inoculated *bri1-6* had a significantly lower DI than the wildtype En-2 (p=0,003; Kruskal-Wallis test; Tab. 3.2) at the high spore concentration (10^7spores/ml). The very low DI values of 50 (combined inoculation) and 67 (clubroot only) of the mutant indicates that these plants were more tolerant to the pathogen in contrast to the wildtype with a DI of 80 (clubroot only). The En-2 wildtype showed a moderate resistance to clubroot in this experiment as well with a DI of 61 in the combined inoculation.

The experiment with the lower spore concentration (10^6 spores/ml) did not yield significant results (Fig. 3.2), the wildtype was slightly less susceptible after *A. alternatum* inoculation but with the mutant no difference was observed.

Table 3.2.: Disease rating of *A. thaliana* mutants after inoculation with *A. alternatum* (+) and *P. brassicae* (-). Abbreviations: DI - Disease Index, avg - average.

Mutant	Spores / ml	Inoculation ± *A. alternatum*	n	DI	Background	n	DI (avg)
wrky18	10^5	+ / -	36 / 31	63 / 59	Col-0	35 / 30	73 / 53
"	10^6	+ / -	33 / 33	96 / 86	"	30 / 30	100 / 100
wrky18oe	10^6	+ / -	66 / 65	75 / 71	"	25 / 24	76 / 70
"	10^7	+ / -	69 / 65	76 / 82	"	31 / 31	83 / 90
bri1-6	10^6	+ / -	30 / 30	73 / 77	En-2	30 / 28	72 / 84
"	10^7	+ / -	33 / 33	50 / 67	"	31 / 33	61 / 80

3.5. Physiological changes in the host upon challenge with the endophytic fungus

Despite the effects observed on the molecular level, physiological traits were recorded in inoculation experiments with the fungus whenever possible. When applicable, the fresh weight of above ground plant parts and the stem length were measured at the timepoint of the disease rating.

3.5.1. A. alternatum affects plant growth

Inoculation of soil and hydroponically grown Arabidopsis with *A. alternatum* caused a slight but reproducible growth reduction in most experiments but increased the biomass of clubroot-infected plants (Fig. 3.15).

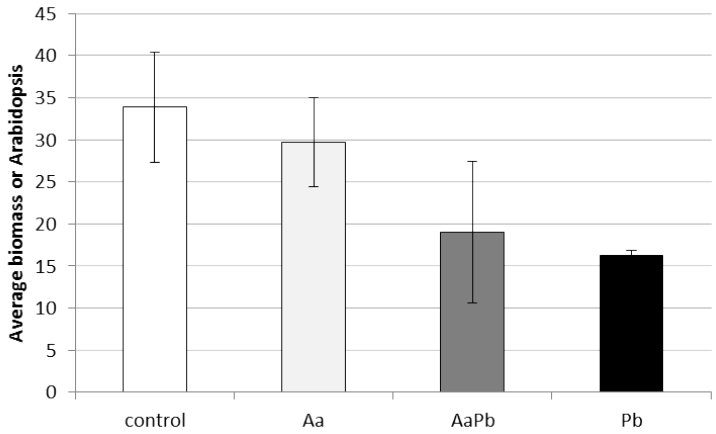


Figure 3.15.: Representative average fresh weight of hydroponic 5 week old Arabidopsis plants 20 days after inoculation with *A. alternatum* and *P. brassicae*. Bars represent standard deviation from 2 independent experiments with n=48 to 55. Abbreviations: Aa - *A. alternatum*, Pb - *P. brassicae*.

In axenically grown Arabidopsis *A. alternatum* promoted the growth of the plants significantly by factor 4 to 5 (Fig. 3.16). This growth promotion had not been reported previously. In addition to an enhanced growth, the plants had longer and thicker stems, more lateral roots and were stiffer compared to the control plants.

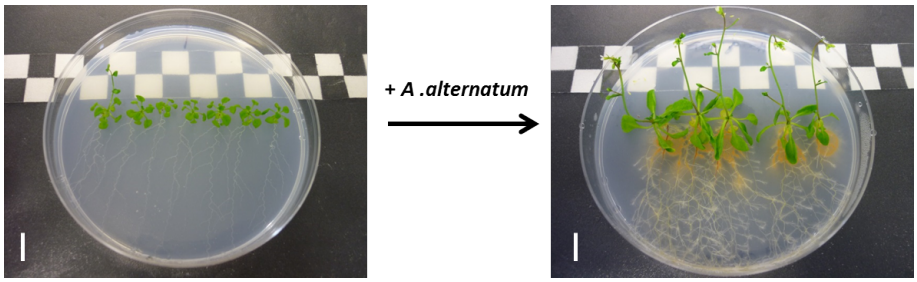


Figure 3.16.: Growth promotion of Arabidopsis after inoculation with *A. alternatum* (10^6spores/ml). The plants on both plates are 4 weeks old and grew under long-day conditions in the climate chamber (8 hours dark at 18°C, 16 hours light at 13°C). Left are Arabidopsis grown on HL medium for 4 weeks without further treatment, right are Arabidopsis 2 weeks after inoculation with *A. alternatum*. Scale bars and black and white squares indicate 1 cm.

The stem lengths of rapeseed plants were measured at the time point of the disease rating 6 to 9 weeks after inoculation with *A. alternatum*. The stems of *A. alternatum*-inoculated rapeseed plants were significantly longer than that of the controls at the time point of the disease rating (Ability: p=0,010 and 0,009; Visby: p=0,019; 2-sided t-test).

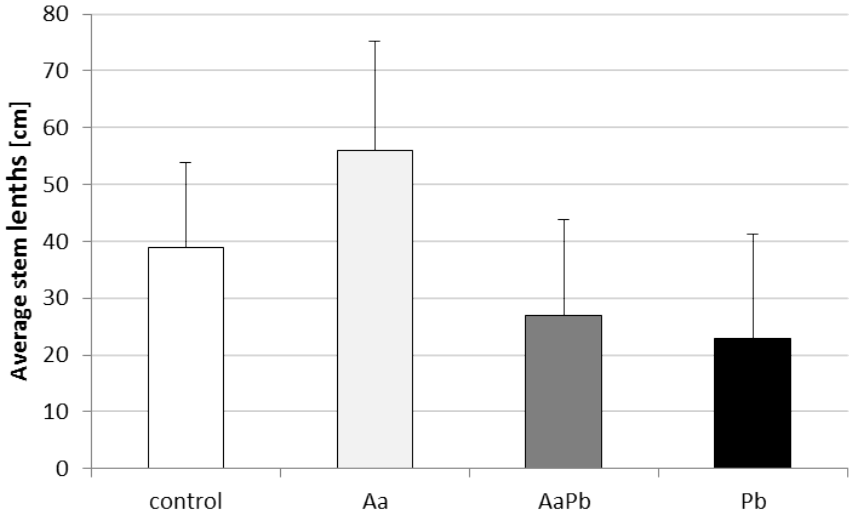


Figure 3.17.: Stem lengths of *B. napus* var. Ability 9 weeks after inoculation with *A. alternatum* (Aa) and *P. brassicae* (Pb). The graph shows representative results for 3 experiments with rape-seed, n=30.

3.5.2. Exploring the role of sugar and hormones in the interaction Arabidopsis-Acremonium

Arabidopsis plants were grown on medium with no added carbon and inoculated with *A. alternatum* (Fig. 3.18 C and 3.19 A).

According to Sun et al. (2014) Arabidopsis plants were grown on 1/4 MS medium and co-cultivated with *A. alternatum* without direct contact for 7 days (Fig. 3.18) on carbon containing and carbon free medium. The presence of the fungus on the same plate increased the biomass of the plants on all media tested though the effect was not as strong as in direct-contact experiments (Fig. 3.19 and Fig. 3.18 B).

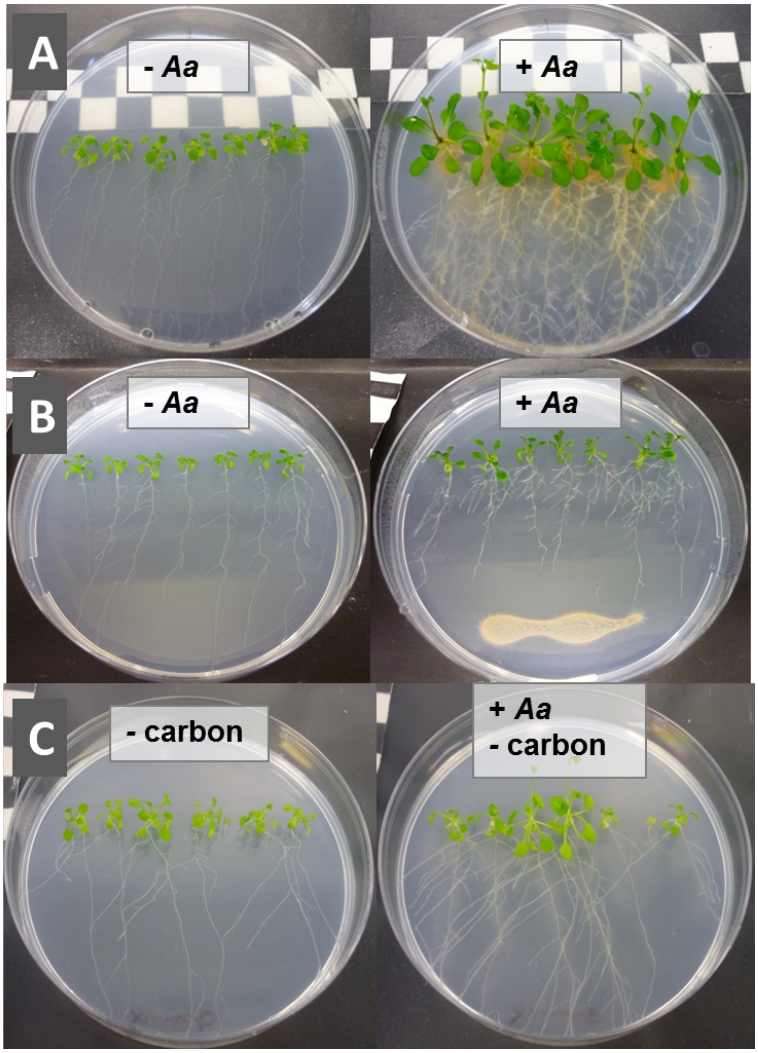


Figure 3.18.: *A. alternatum* (*Aa*) promotes the growth of Arabidopsis in axenic cultures upon direct and indirect contact with the fungus (A, B) as well as on carbon free medium (C). Plants were grown on plant medium with 1% saccharose (A,B) or without carbon (C) and inoculated with *A. alternatum* (10^6spores/ml) directly on the roots (A, C) or indirectly by pipetting 100 μl spore suspension on the plates (B). Pictures show the plants 1 (B) or 2 weeks after inoculation (A, C).

In the indirect interaction the number of lateral roots was determined as well. Upon co-cultivation, Arabidopsis produced significantly more lateral roots than the control plants (Fig. 3.19 B). This suggests an involvement of auxin in the growth promotion.

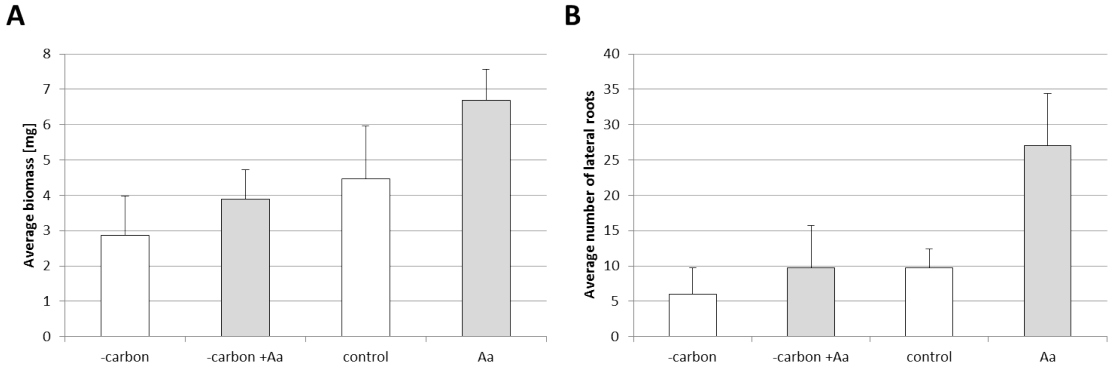


Figure 3.19.: Indirect interaction of Arabidopsis with *A. alternatum* (Aa). The fungus promoted growth of Arabidopsis by 36% on carbon-free medium and 50% on the control medium. Inoculated plants had 59% more lateral roots than controls on carbon free and 174% more on the control medium.

Three receptor mutants of Arabidopsis (*tir1*; *afb1-3*; *afb1-2,2-3*), defective in the perception of auxin, were cultivated on agar plates with HL and inoculated with *A. alternatum*. All 3 mutants showed a growth promotion after inoculation with the endophyte (by factor 2 to 3) that was similar to the corresponding wildtype plants (see Fig. 3.20 for growth promotion of 1 representative auxin receptor mutant).

To further assess the role of auxin in the observed growth promotion, the auxin transport inhibitor NPA was included in the growth medium at a concentration of 10 µM. Plants grown on this medium showed a phenotype similar to *aux-1* mutants with agravitropically growing roots. Addition of NPA resulted in the lowest growth promotion of all experiments (19%) which indicates that *A. alternatum* might manipulate the auxin transport to facilitate plant growth (Fig. 3.20). Unfortunately no results were obtained with *aux-1* mutants due to contamination issues.

Additionally hormone mutants and overexpressors for cytokinin (*cyr1*, *ckx1*), ethylene (*eto2*, *eto3*), ABA (*aba1*), jasmonate (*jar1*) and brassinosteroids (*bri1-6*) were inoculated with *A. alternatum*. The list of all tested mutants and their description can be found in section 2.3. The brassinosteroid biosynthesis inhibitor propiconazole was applied on Arabidopsis before inoculation with *A. alternatum*. All hormone mutants and the corresponding wildtypes as well as plants treated with the hormone blocker showed a growth promotion when inoculated with *A. alternatum* (Fig. 3.20). The amplitude of the response to the fungus was quite different though and ranged from roughly 70 to 350% (Fig. 3.18).

3.5.3. Senescence and flower production

Inoculation with *A. alternatum* caused a shift in senescence in many experiments. Typically, plants on agar plates inoculated with the endophyte flowered earlier and produced more flowers than control plants (see figure 3.21 A).

In rapeseed the early flowering was highly significant in the 1 experiment where plants

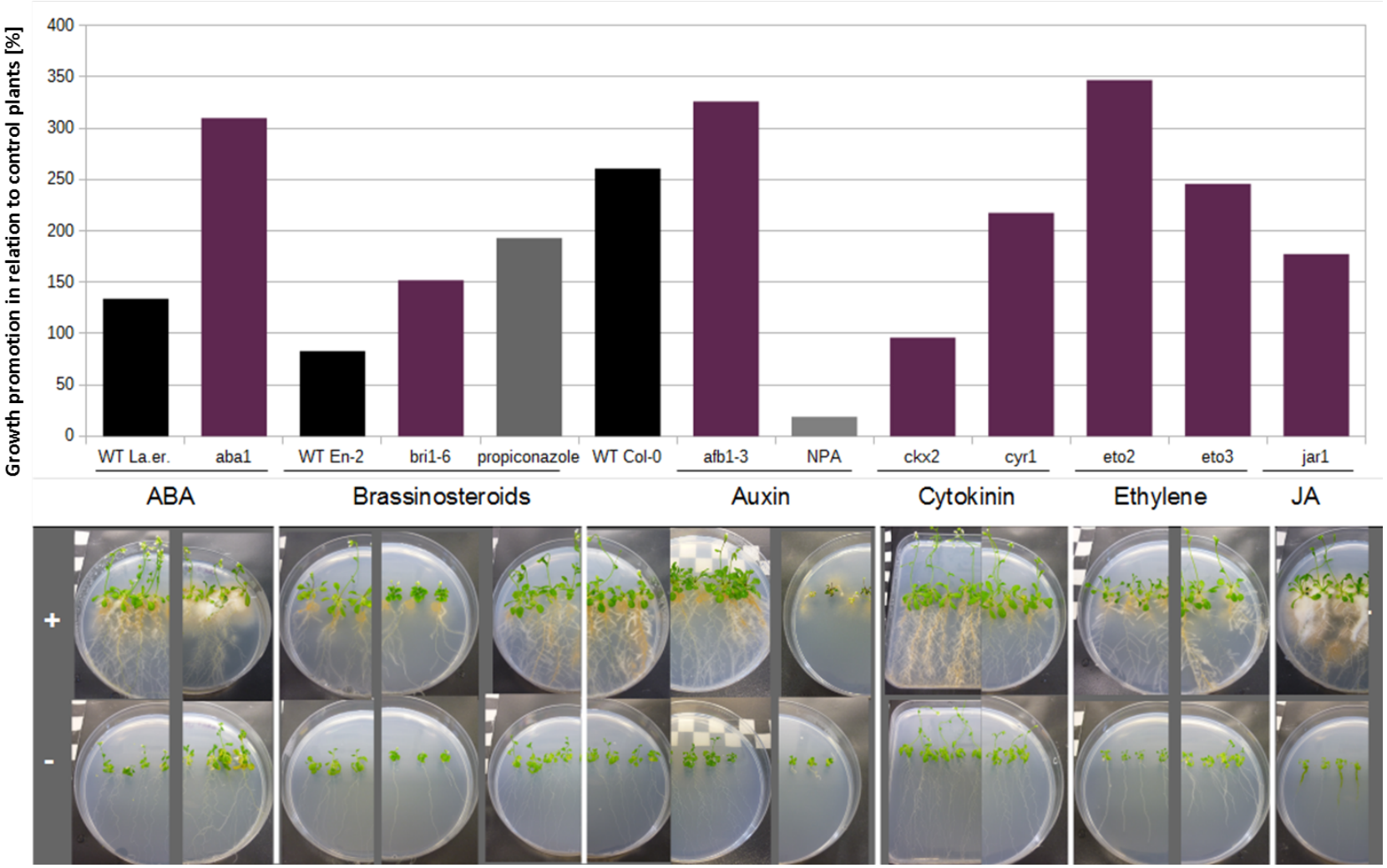


Figure 3.20.: Growth promotion induced by *A. alternatum* in Arabidopsis mutants and the corresponding wildtypes (WT) and after treatment with hormone blockers in relation to untreated plants. Arabidopsis mutants were grown on HL medium for 2 weeks before inoculation with *A. alternatum*. All experiments were terminated 2 weeks after inoculation. NPA was added to the medium at a concentration of 10μM, wildtype plants were treated with 10 μM propiconazole 1 week before inoculation with *A. alternatum*. Black bars indicate the respective wildtype, violet bars are mutants, grey bars are hormone blockers. A plus (+) indicates inoculation with *A. alternatum*, minus (-) are control plants.

produced flowers (t-test; p=0,0003). With regard to flower production in rapeseed a drastic increase in seed production was achieved under greenhouse conditions, resulting in almost 10 times more seeds than the control plants (n=14, Fig. 3.21 B, Auer and Ludwig-Müller (2014)).

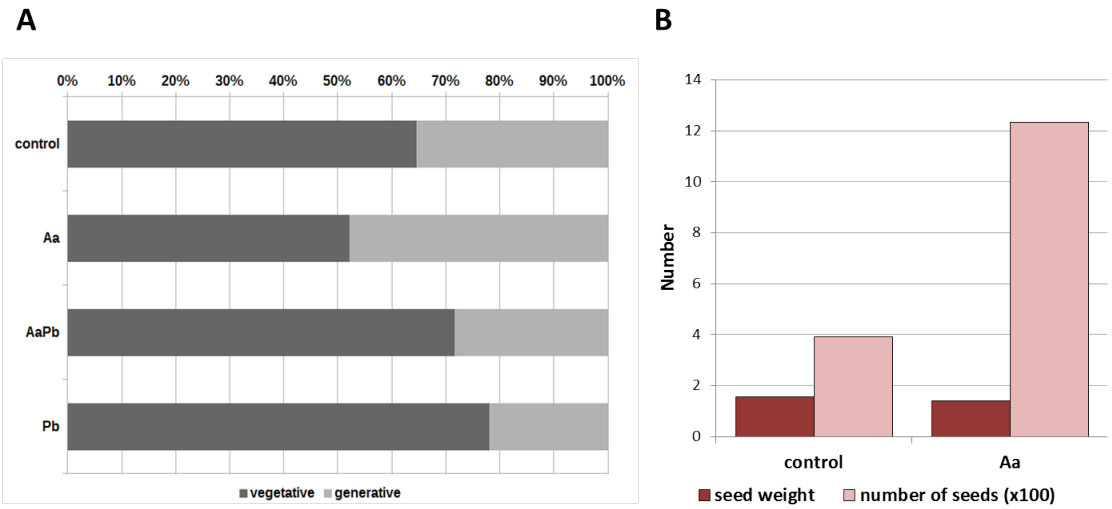


Figure 3.21.: Growth stages of Arabidopsis plants treated with *A. alternatum* and *P. brassicae* and seed yield of *Brassica.* (A) Hydroponically grown Arabidopsis treated with viable spores (10^7/ml) of *A. alternatum* (Aa) and *P. brassicae* (Pb) at 9 dai; 3,5 weeks old. Representative results of 1 experiment are shown with n=75-91, (B) Seed yield of *Brassica napus* inoculated with *A. alternatum* (Aa).

3.6. The potential of A. alternatum as biocontrol agent

In this study the fungus was tested for its potential to solubilise phosphate and fix nitrogen. Furthermore, a crude cell wall extract (CWE) and autoclaved spores of the fungus were applied on Arabidopsis and rapeseed to test whether this treatment could reduce clubroot symptoms.

3.6.1. Autoclaved spores and a crude CWE from A. alternatum reduce clubroot symptoms in Arabidopsis and Brassica

The application of a crude CWE and autoclaved spores of *A. alternatum*, here termed "priming solutions", applied 3 to 4 days before inoculation with *P. brassicae* resulted in a reduction of clubroot symptoms in the tested *B. napus* var. Ability and in Arabidopsis. Due to time and material restrictions the experiments were carried out once.

3.6.1.1. Disease rating

In Arabidopsis the pre-treatment with priming solutions and *A. alternatum* reduced the DI significantly in all groups (p-values for CWE, autoclaved spores and *A. alternatum* were

0,004; 0,038; 0,000; Kruskal-Wallis test against *P. brassicae* treatment; Fig. 3.22 A). However, the plants clearly suffered from the inoculation with *P. brassicae* and had a lower biomass than control plants (Fig. 3.22 B, C).

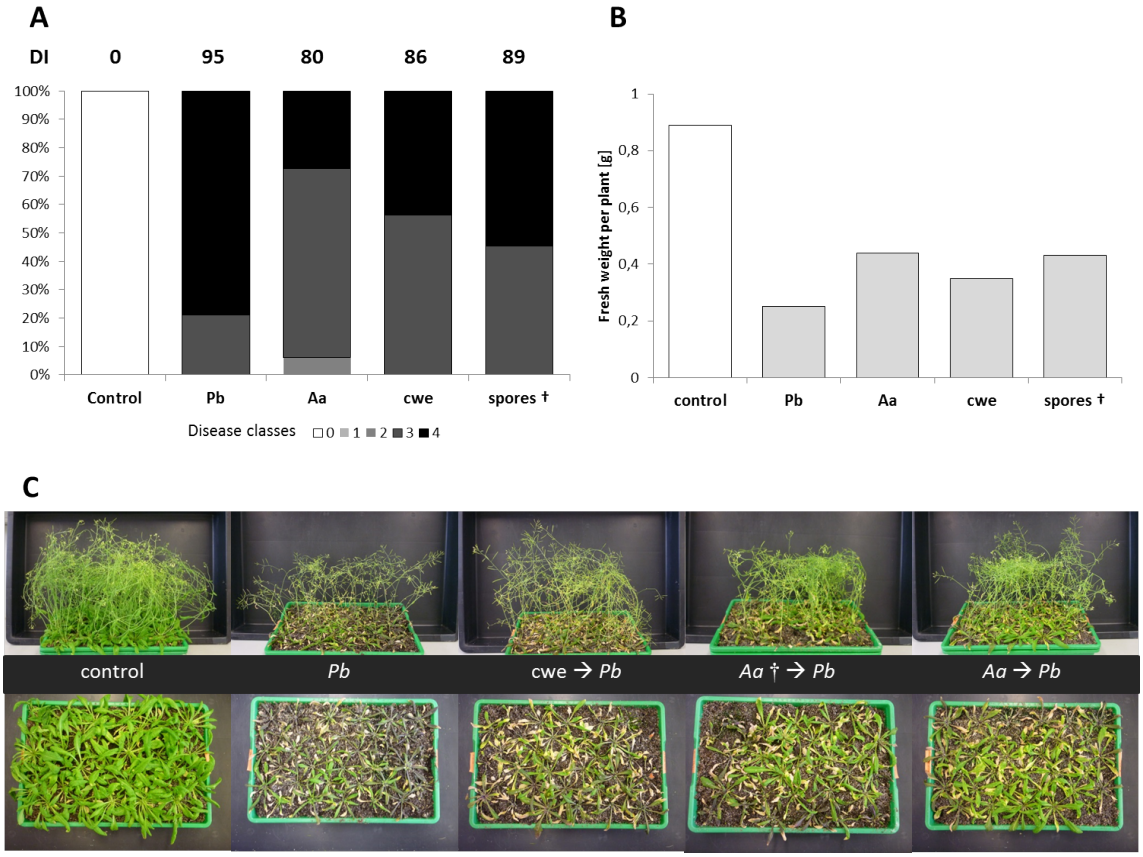


Figure 3.22.: Disease rating of Arabidopsis Col-0 pre-treated with priming solutions and *A. alternatum* before inoculation with *P. brassicae*. A) Disease index (DI), B) fresh weight of above ground plant biomass, C) plants at the time point of the disease rating, 28 days after inoculation with *P. brassicae*. Pre-treatment was done 4 days prior to inoculation with *P. brassicae* with 4 ml of a 1% crude cell wall extract solution (CWE), 2 ml of autoclaved *A. alternatum* spores (spores †) and 2 ml *A. alternatum* spores. The spore concentration was 10^6/ ml for each treatment and n=33 plants per treatment.

Pre-treatment of rapeseed with the priming solutions led to a lower disease index and that was significant in the 4% CWE treatment (p=0,028; Kruskal-Wallis test; see Fig. 3.23 A). While the clubroot infection reduced the biomass of the plants significantly (p=0,00004; t-test; 3.23 B), the application of priming solutions had a positive effect on the biomass. Plants in the high disease categories (3 and 4) were significantly larger than clubroot infected plants without further treatment (Fig. 3.23 C) .

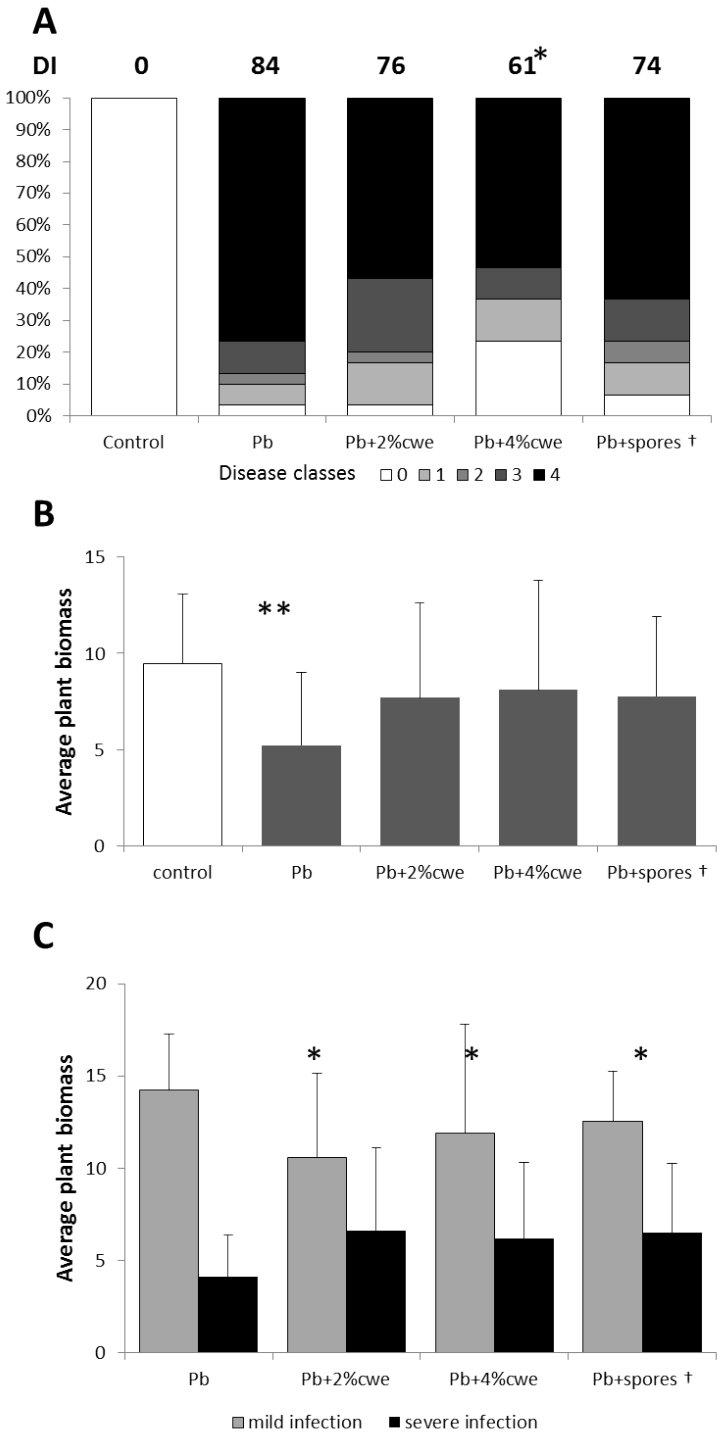


Figure 3.23.: Effects of priming solutions on disease parameters in *B. napus* var. Ability. Plants were inoculated with CWE or autoclaved spores of *A. alternatum* (2×10^6 spores/ml) 4 days before inoculation with *P. brassicae* (2×10^6 spores/ml). A) Disease index; pre-treatment with 4% CWE yielded significantly less galls (p=0,015, t-test) than the other treatments. B) Average fresh weight of plants; inoculation with *P. brassicae* resulted in significantly smaller plants (t-test control vs. Pb; p=0,00004) whereas with the other treatments plant weight was similar to the controls (t-test control vs. treatment; p=0,114; 0,272; 0,095). C) Average biomass of plants rated as mildly infected (disease classes 1 and 2) and severely infected (disease classes 3 and 4); the pre-treatment increased the overall plant biomass significantly (t-test Pb vs. pre-treatment; p=0,034; 0,024; 0,015). Error bars are standard deviations and n=30 plants per treatment. Abbreviations: CWE - cell wall extract, Pb - *P. brassicae*, DI - disease index.

The average biomass of the priming solution-treated plants was slightly lower than that of controls but not statistically different from the control plants (n=30 per treatment; p>0,095), in contrast to *P. brassicae* infected rapeseed plants that had significantly lower average biomasses than the controls (p=0,0004, Fig. 3.23 B).

3.6.1.2. Light Microscopy of roots pre-treated with 4% CWE

Samples of disease rated *B. napus* roots var. Ability that had been pre-treated with the priming solutions were prepared in Technovit and cut with a microtome in 5 to 7 µm thick slices. The samples were stained with cotton blue to stain plasmodia and counter-stained with safranin O to visualise plant cell walls. 2 to 3 independent samples were investigated per treatment.

Samples of all treatments from the high disease categories where the respective roots had been rated as 3 or 4 were compared. In samples treated with a high amount of CWE (4%) the size of cells containing plasmodia and resting spores were similar to the size of normal cells in many cases (Fig. 3.24). This reduction of cell size correlated with smaller galls in the high disease categories in comparison to not pre-treated plants.

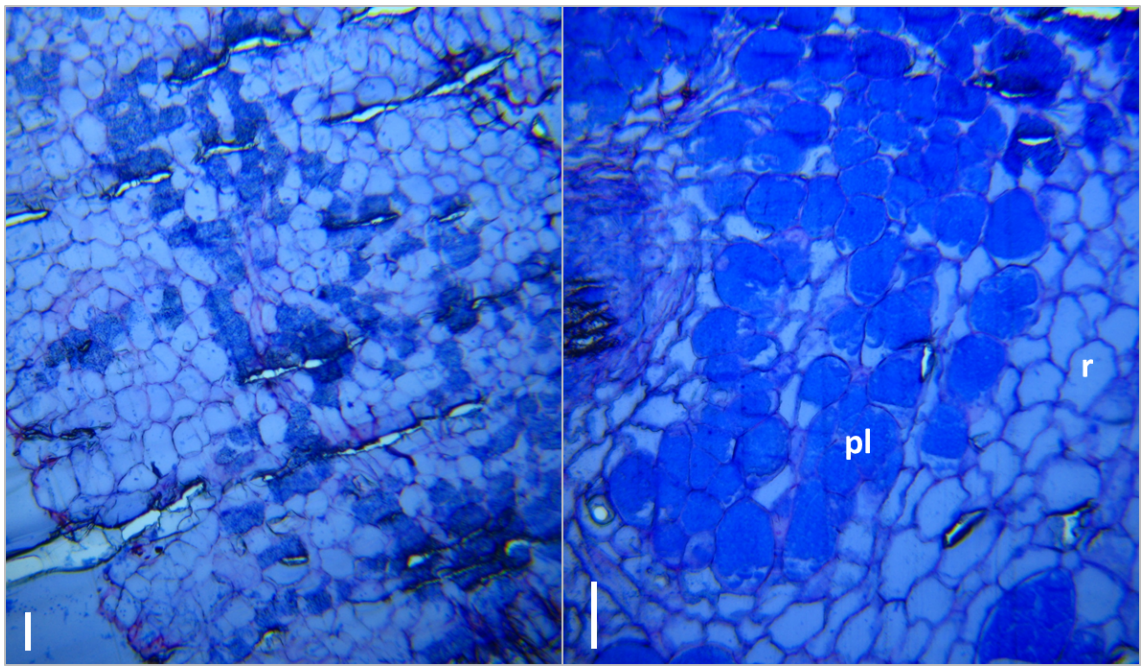
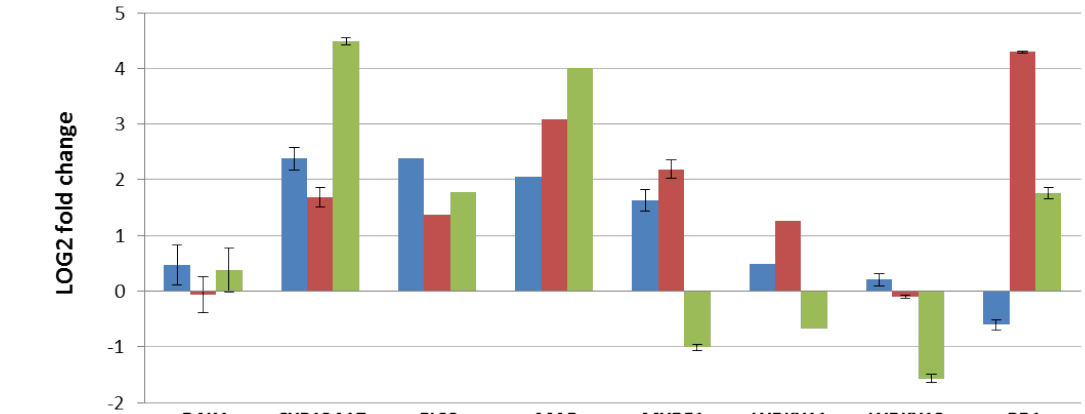


Figure 3.24.: Light microscopy images of *B. napus* var Ability roots pre-treated with 4% CWE 6 weeks after inoculation with *P. brassicae.* Pre-treatment of plants with CWE results in plasmodia containing cells with sizes similar to uninfected cells. Root tissue was embedded in technovit and stained with cotton blue and safranin O. Abbreviations: CWE - cell wall extract, pl - plasmodia, r - uninfected root cell. Scale bars are 10 μm.

3.6.1.3. Altered trancripts levels in roots after treatment with priming solutions

Arabidopsis Col-0 were grown on Hoagland medium in agar plates for 2 weeks and then inoculated at the roots with the priming solutions CWE (1%), autoclaved and living spores of *A. alternatum* (10^7 spores/ml). Three dai the plants were harvested and RNA was extracted from the roots. In another experiment Arabidopsis Col-0 was inoculated with *A. alternatum* and the roots harvested 1 and 4 dai. For both experiments qRT-PCR was performed with the MAMP-related gene primers and *PR1*.



	BAK1	CYP12A17	FLS2	MAP	MYB51	WRKY11	WRKY18	PR1
cwe	0,47	2,38	2,39	2,06	1,63	0,49	0,21	-0,6
dead spores	-0,06	1,69	1,37	3,08	2,19	1,26	-0,1	4,3
living spores	0,38	4,49	1,78	4	-1,01	-0,67	-1,57	1,76

Figure 3.25.: Relative expression of MAMP-related genes and *PR1* from Arabidopsis roots 3 dai with priming solutions of *A. alternatum* (10^6 spores/ml). Arabidopsis were grown on HL medium for 14 days, inoculated with *A. alternatum* and harvested 3 days after inoculation. SD values are shown for experiments with 2 PCRs. Abbreviations: CWE - cell wall extract, dead and living spores refer to *A. alternatum* spores.

Treatment with the priming solutions resulted in transcript changes of all MAMP-related genes except *BAK1*. *CYP12A17*, *FLS2* and *MAP* were induced in all treatments (Fig. 3.25). The level of induction was similar for all 3 genes after CWE treatment (LOG2 fold change 2,38; 2,39; 2,06) and *MAP* and *CYP12A17* showed the highest induction upon challenge with the living fungus (LOG2 fold change 4,00; 4,49). *MYB51* was induced after treatment with the priming solutions (LOG2 fold change 1,63; 2,19) and downregulated after *A. alternatum* inoculation (LOG2 fold change -1,01). *WRKY11* showed a response similar to *MYB51* but with a lower amplitude (Fig. 3.25). *WRKY18* did not respond to the priming solutions but was downregulated after endophyte challenge (LOG2 fold change -1,57). *PR1* was strongly induced upon treatment with autoclaved spores and weaker at the living-spore treatment (LOG2 fold change 4,30; 1,76).

A comparison of these data with the expression profiles of the same genes in the hydroponic culture experiments (section 3.3.1.2, Fig. 3.8) reveals a different response of some genes after *A. alternatum* inoculation.

Inoculation with *A. alternatum* at the 2 time points 1 and 4 dai confirmed the data for *BAK1*, *CYP12A17* and *WRKY11* from the other experiments to some extent (Fig. 3.26). Especially the cytochrom P450 gene *CYP12A17* showed a constant trend in these experiments - it was upregulated in the microarray and both plate assays at the time points 1, 3 and 4 dai.

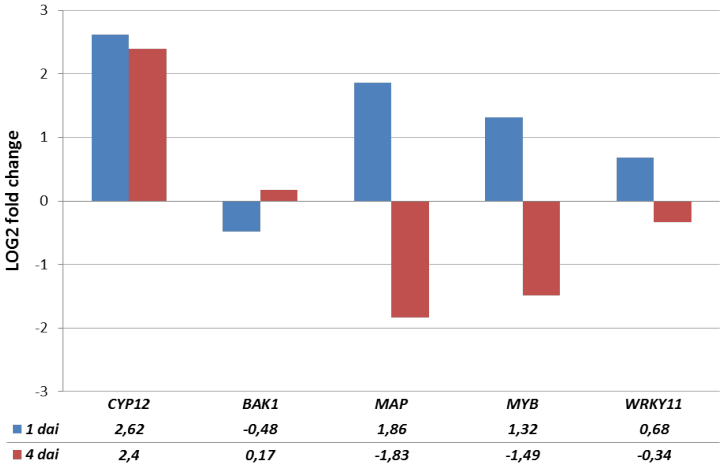


Figure 3.26.: Expression profile of MAMP-related genes from Arabidopsis roots inoculated with *A. alternatum* 1 and 4 dai. Data were obtained from qRT-PCR of root RNA. Arabidopsis were grown on HL medium for 14 days, inoculated with *A. alternatum* and harvested 24 and 96 hours after inoculation.

3.6.2. Enzymatic activities of A. alternatum in vitro

3.6.2.1. Phosphate solubilisation

A. alternatum was grown on phosphate containing media. A clearing zone was observed on PCa medium that contained calcium phosphate but not on the medium containing iron phosphate (Fig. 3.27). According to Ogbo (2010) the solubility of phosphates is in the order Ca > Al > Fe. Since *A. alternatum* is able to solubilise the more soluble calcium phosphate but not iron phosphate the fungus is a rather weak phosphate solubiliser. The experiment was repeated once with the same results.

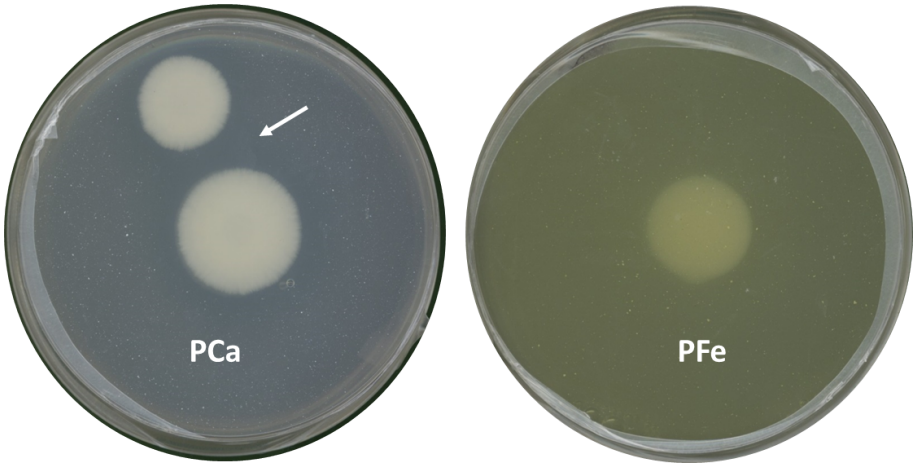


Figure 3.27.: Performance of *A. alternatum* as phosphate solubiliser. The left side shows the fungus on calcium phosphate containing medium (PCa). The arrow indicates the border of the clearing zone of phosphate particles that spreads from the centre of the fungal colony until beyond its growing borders. Right: the fungus on iron phosphate (PFe) with no clearing zone, white phosphate particles are still visible under the colony and around it..

3.6.2.2. Nitrogen fixation

NFb medium is used in studies with bacteria to assess the abilities of the microbes to fix nitrogen (Forchetti et al. 2007; Zheng et al. 2011). Organisms that are able to fix atmospheric nitrogen can reduce nitrogen to NH3+ which leads to a change in pH and respectively to a colour change of the medium from green to blue due to a pH indicator in the medium. Cultivation of *A. alternatum* on NFb medium led to a colour change from green to blue (Fig. 3.28). However, to date no fungi are known that can fix atmospheric nitrogen. Since no positive or negative controls were carried out for this experiment to confirm the nitrogen fixation the results are doubtable.

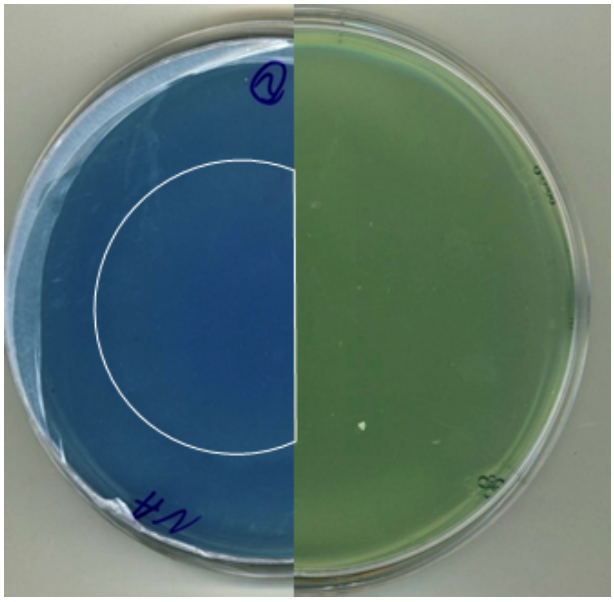


Figure 3.28.: Nitrogen fixation experiment with *A. alternatum* on NFb medium. Left: After cultivation of *A. alternatum* on NFb the medium turns blue, the half-circle indicates the growing zone of the fungus. Right: control medium without fungus for comparison. The photos were taken by Mira Zharova.

3.6.3. Salt and osmotic stress in Arabidopsis

Inoculation of Arabidopsis with the endophyte on plates with a mild concentration of sodium chloride or mannitol had a positive effect on the growth of plants. The control plants remained small and flowered early while plants treated with *A. alternatum* showed a delay in flowering (data not shown) and were larger (Fig. 3.29 A).

The endophyte promoted the growth of plants by factor 5 to 7 in plants that were exposed to NaCl and by factor 0,5 by those exposed to mannitol (Fig 3.29 B).

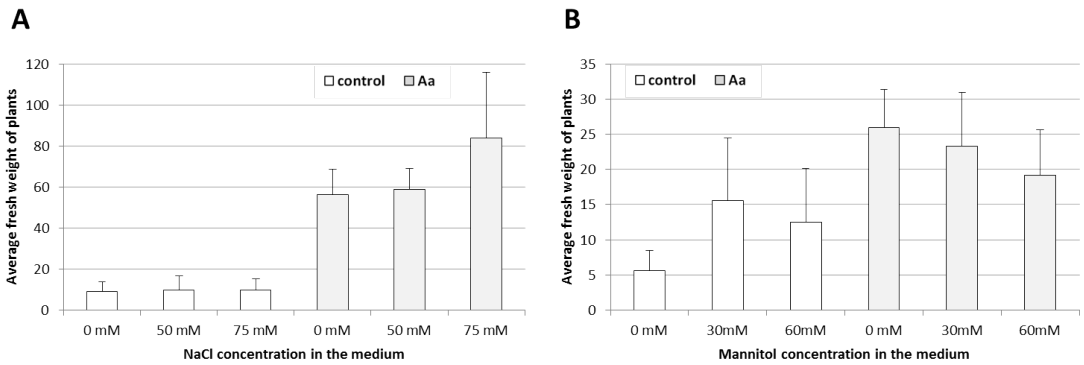


Figure 3.29.: *A. alternatum* reduces the osmotic and salt stress and increases the biomass of Arabidopsis. Arabidopsis were grown on HL medium containing mild concentrations of NaCl (50 mM, 75 mM) for 1 week (A) or HL with mannitol (30 mM, 60 mM) for 2 weeks (B), inoculated with *A. alternatum* (10^6 spores/ml) and harvested 14 days later.

3.6.4. Antagonistic activity of A. alternatum

So far, no studies have been conducted for *A. alternatum* on the biochemical potential of this fungus. Therefore, the enzymatic activity of the endophyte was tested *in vitro* and in fungus vs. fungus interaction tests.

The *in vitro* activity of the fugnus for the production of cellulase, protease and chitiniase were tested. Each experiment was done with 5 replicates. None of the *in vitro* tests yielded positive results for an enzymatic activity of the fungus (data not shown). It was therefore concluded that the fungus is not able to produce protease, chitinase or cellulase.

In another experiment the endophyte was tested against other phytopathogenic fungi on a fungus vs. fungus plate assay. The endophyte did not show a strong competitive behaviour and was overgrown by most of its interaction partners (*Aspergillus niger*, *Fusarium graminearum*, *Macrophomina phaseolina*, *Schizophyllum commune* and *Trametes quercina*; data not shown).

In some cases the growth of both fungi was intermingling with *A. alternatum* slowing its growth to some extent which made it hard to decide whether the endophyte was overgrown by the antagonistic fungus or not. This was the case on some but not all of the plates with *Fusarium culmorum*, *F. avenaceum*, *Bjerkandera adusta*, *Gloeophyllum sepiarium* and *Trichoderma* (Fig. 3.30). A slight inhibition with a small inhibition zone between both interaction partners was observed with *Piriformospora indica* and (Fig. 3.30). For *Cladosporium* 2 types of interaction were observed - mutual inhibition and overgrowth by *A. alternatum* (Fig. 3.30).

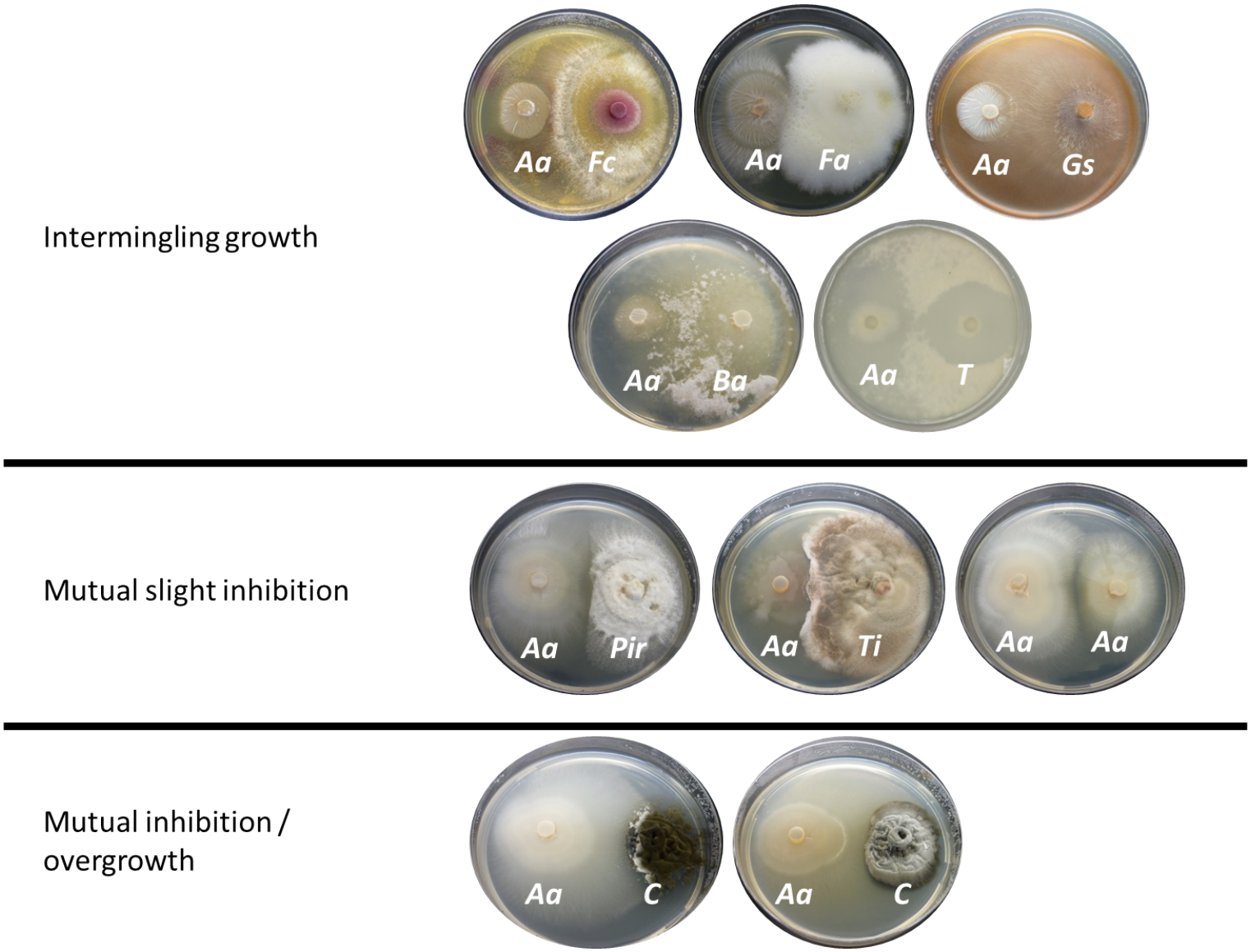


Figure 3.30.: Interaction of *A. alternatum* with phytopathogenic fungi. *A. alternatum* was grown on PDA or MEA medium 1 week before the interaction partner was placed in 4 cm distance on the same plate. All plates were kept at 26°C in the dark. Pictures were taken 2 months after transfering. Abbreviations: *Aa - A. alternatum*, *Fa - Fusarium avenaceum*, *Gs - Gloeophyllum sepiarium*, *Ba - Bjerkanda adusta*, *T - Trichoderma* spec., *Pi - Piriformospora indica*, *Ti - Talaromyces islandicus*, *C - Cladosporium* spec.. The type of interaction is classified according to Skidmore and Dickinson (1976).

4. Discussion

4.1. Alternative methods to control clubroot - could an endophyte be the answer?

Natural means to suppress pathogens by biocontrol agents (BCAs) such as soil bacteria and fungi have a long tradition especially in developing countries and are often more cost-efficient and environmentally friendly than agrochemicals (Bettiol 1996; Azevedo et al. 2000). However, the variation in terms of a successful control of plant pests is still a considerable drawback when using these agents (Romero et al. 2003; Peng et al. 2014). In addition to unfavourable abiotic conditions that can decrease the effectiveness of biocontrol other microbes also influence the outcome of the interaction between beneficial partners (Green et al. 1999).

Despite the relatively wide usage of beneficial soil microbes to achieve pest control the underlying mechanisms that lead to a suppression of diseases are mostly poorly understood or have not been studied yet (Pieterse et al. 2014). Especially molecular studies on the relationship of plants and beneficial endophytic fungi are rare and poorly understood.

So far, 2 beneficial fungi that promote plant growth and protect their host plant against pathogens haven been studied in more detail: *Trichoderma* sp. and *Piriformospora indica* (Waller et al. 2005; Woo et al. 2006; Van Wees et al. 2008). *Trichoderma* species can induce systemic acquired resistance and rely on plant-derived carbon to establish their mutualistic relationship with the plant (reviewed in Pieterse et al. 2014). They utilise auxin signaling pathways to enhance the biomass of plants but the mechanism of resistance induction is still unclear (Pieterse et al. 2014). *Piriformospora indica* induces resistance mechanisms in barley independent of salicylic acid, jasmonate and ethylene (Waller et al. 2005) and by utilising jasmonate-dependent resistance pathways and NPR1 in Arabidopsis (Stein et al. 2008). A cell wall extract of *Piriformospora indica* induces the phosphorylation of MAP kinases which induce defence responses in the plant (Vadassery and Oelmüller 2009).

Several attempts have been made to use BCAs to control the clubroot pathogen *Plasmodiophora brassicae* (Narisawa et al. 2000; Cheah et al. 2001; Peng et al. 2011; Lahlali et al. 2013). The complex life cycle of this obligate biotroph pathogen makes the control of this disease rather difficult. A root colonising microbe could provide permanent and long lasting control as recently shown for the potential endophytic strain *Heteroconium*

chaetospira in greenhouse trials of rapeseed (Lahlali et al. (2014)). Under low pathogen pressure (2×10^5 spores/pot) *H. chaetospira* controlled clubroot completly but under a moderate spore concentration of *P. brassicae* (2×10^6spores/pot) the endophyte had no effect on clubroot symptoms in rapeseed. This shows that biocontrol of clubroot by the means of beneficial microbes is generally possible but likely dosage-dependent. A high spore concentration of the endophytic fungus *Acremonium alternatum* (10^7spores/ml) reduced clubroot symptoms in Arabidopsis and Chinese cabbage by 40% at moderate pathogen concentrations (10^6 spores/ml) and it was hypothesised that the fungus triggers resistance genes in the plant (Doan et al. 2010; Jäschke et al. 2010)

In this study different concentrations of *A. alternatum* were tested against the clubroot pathogen *P. brassicae* in Arabidopsis and 2 local German rapeseed cultivars. The resistance of plants was assessed qualtitatively by determining physiological parameters such as the disease index and the biomass of plants.

The main focus of this work though was on the quantitative resistance responses of Arabidopsis and rapeseed roots. For this RNA was extracted from infected plant roots and examined with quantitative RT-PCR for the transcription levels of resistance genes.

4.1.1. Clubroot symptom reduction in Arabidopsis

The fungus *Acremonium alternatum* is a grass endophyte with biocontrol potential that reduced the disease severity of the clubroot disease caused by the biotroph protist *Plasmodiophora brassicae* (Breen 1994; Jäschke et al. 2010; Doan et al. 2010). The co-inoculation with the fungal endophyte led to a significant reduction of disease symptoms in Arabidopsis wildtype plants of 6 to 24% according to the disease indices (DI). This disease reduction is lower than the reported 40% from Jäschke et al. (2010). The discrepancy is likely the result of differing spore concentrations since Doan et al. (2010) and Jäschke et al. (2010) used a lower concentration of *P. brassicae* of 10^6 spores/ml for their experiments and for *A. alternatum* 10^7 spores/ml.

In the here presented study an equal concentration of both pathogen and endophyte spores was used in all experiments. The high spore concentration (10^7spores/ml) achieved the best biocontrol effect, which confirms the findings of both earlier studies. Pre-treatment with *A. alternatum* did not result in a lower DI in Arabidopsis compared to the application at the same time point together with *P. brassicae*, which was also observed in Jäschke et al. (2010).

However, when using BCAs whose effectiveness depend on induced resistance mechanisms of the plant the time point of the application of these BCAs is crucial for the success of the treatment. Because of the nature of inducible plant defences there is a time-lag between the perception of a pathogen effector and the expression of defence mechanisms and in between the plant is vulnerable to invaders (Vos et al. 2013, see also section 4.1.2).

The time point of the application of BCAs is only one factor for an efficient pest control, others are soil properties such as moisture and pH, the concentration of the pathogen in the soil and favourable abiotic conditions (Romero et al. 2003). In the present study some variation was observed regarding the reduction of the clubroot disease. In experiments with a low pathogen pressure (10^5 spores/ml) the disease index of the co-inoculated plants was higher than that of the plants infected with clubroot only. This inconsistency has been reported for other biocontrol agents as well (e. g. in Romero et al. 2003; Peng et al. 2014) and is one of the drawbacks when using beneficial microbes. To circumvent this problem and be able to predict the success for the application of microbes a set of desirable traits has been drafted that every beneficial microorganism should possess in order to be used in pest management (Gaiero et al. 2013; Lorito et al. 2014). Some of these traits were also tested in this study for *A. alternatum* (see section 4.6).

4.1.2. Clubroot symptom reduction in B. napus

The fungus *A. alternatum* reduced the DI in rapeseed at a high pathogen pressure by 19%. Doan et al. (2010) found a reduction of 40% for *Brassica rapa* (Chinese cabbage) with the ratio 10^7 vs. 10^6 spores/ml for *A. alternatum* vs. *P. brassicae* (see previous section for this). The summer rapeseed variety "Ability" was more susceptible than the winter variety "Visby". For *Brassica* is has been reported that the susceptibility to clubroot is race-specific and also depends on the *P. brassicae* isolate (Werner et al. 2008; Diederichsen et al. 2009). The intricate interaction the clubroot pathogen forms with its host is highly specific and it is known that the pathogen adapts quickly to its environment and produces new, more virulent pathotypes (Diederichsen and Sacristan 1996). This is one of the major problems for the breeding of resistant cultivars as single-gene resistance is overcome rather rapidly on the field within a few growing periods (Peng et al. 2011).

From the results with rapeseed it was concluded that viable spores of *A. alternatum* are not suitable for sufficient clubroot suppression of field cultivars.

On the other hand, pre-treatment with a high amount of crude cell wall extract (CWE) from the fungus before pathogen inoculation reduced the disease severity significantly by 28 % and lowered the DI to a level of moderate resistance (DI = 61 for 4% CWE). Microscopy revealed that the lower DI correlated with a smaller size of plasmodia containing cells. This fits nicely to the findings that the endophyte enhanced the overall health and survival rates of plants (Auer and Ludwig-Müller 2014). It would be of high interest to identify the active compound of the fungal CWE that attributes to the biocontrol effect.

The fungus induces priming-related responses also in *B. napus* and therefore improves the coordination of plant defence mechanisms to clubroot. The CWE of the fungus likely contains one or more fungal effectors that trigger priming-related responses as shown with studies on Arabidopsis (see Fig. 3.25), however, this was not confirmed for rapeseed yet.

A. alternatum did also promote stem lengths of *Brassica* significantly and increased the seed yield of rapeseed plants by 68 % but not the seed weight. A longer stem is advantageous especially for field plants which stand very close to neighbouring plants and thus might suffer from shade effects. In conclusion it can be said that the early application of a crude CWE of *A. alternatum* is beneficial against clubroot and could be used in integrative pest management strategies in combination with other pesticides.

4.2. Priming of the plant against P. brassicae

The main aim of this study was to find out whether the endophytic fungus *A. alternatum* induces resistance genes in Arabidopsis roots which lead to a reduction of symptoms during infection with the clubroot pathogen *P. brassicae*. It was hypothesised that *P. brassicae* represses the defence strategy of the plant but there is a knowledge gap in how the clubroot pathogen evades detection by the host specifically and thus represses plant defence (Siemens et al. 2006; Agarwal et al. 2011). By using quantitative RT-PCR and by means of a microarray it could be shown how the clubroot pathogen manipulates the first line in plant defence and that co-inoculation with the endophyte enhances the immune response of the plant against clubroot.

4.2.1. A. alternatum circumvents the suppression of pathogen recognition by P. brassicae

One of the major findings in this work are the early responses triggered by the endophyte that likely facilitate detection of the clubroot pathogen. In the primary infection, zoospores of *P. brassicae* infect root hairs and build plasmodia around 4 dai which then form a zoosporangium around 5 to 6 dai (Kageyama and Asano 2009). A microarray analysis during the primary infection (3 dai) revealed that several microbe-associated molecular pattern (MAMP)-responsive genes are activated after co-inoculation with *A. alternatum*: the cytochrome P450 gene *CYP71A12*, a yet uncharacterised nodulin-like *MAP* gene, 2 pathogen recognition receptor genes *(BAK1, FLS2)* and 3 transcription factors: *WRKY11*, *WRKY18* and *MYB51*. However, the induction level of these genes was below the cut-off value of 1 (LOG2 fold change) except for *BAK1* (see Fig. 3.8). These rather subtle changes are typical for priming responses as the pathogen-triggered immunity (PTI) relies on relatively weak signals (see also Fig. 1.3 in section 1.4, Jones and Dangl 2006).

Priming is a cost-effective way for the plant to activate inducible defence-responses upon pathogen attack. After perceiving a priming-relevant inducer the plant prepares to activate inducible defence mechanisms and the state of enhanced alertness leads to a faster and stronger activation of defence mechanisms in comparison to non-primed controls (Vos et al. 2013). The biomass of primed plants was lower than that of the non-primed controls, another indicator for biochemical changes of the host (Vos et al. 2013).

In the samples treated with clubroot, 3 MAMP-related genes were downregulated (*FLS2*, *MYB51*) or not regulated (*WRKY11*) which is clear proof that i) *P. brassicae* actively manipulates the pathogen recognition mechanisms of the plant at this early stage and ii) the beneficial effect against the clubroot pathogen can be attributed to the reactivation of pathogen recognition to some extent.

BAK1 is a coreceptor of FLS2 and both proteins form an active complex upon pathogen recognition thus restricting further colonisation of the respective invader (Jones and Dangl 2006; Heese et al. 2007; Dodds and Rathjen 2010). The low transcript level of *FLS2* (LOG2 fold change -0,39) by *P. brassicae* suggests that this early in the interaction PTI is hindered and thus likely clears the way for a further colonisation by the protist. The plant might therefore compensate this with an increase in transcript levels of *BAK1* as evidenced by the strong upregulation of this gene in clubroot samples. Despite its role as coreceptor of the BRASSINOSTEROID RECEPTOR 1 (BRI1), BAK1 is a positive regulator of MAMP-mediated signaling in Arabidopsis. This was demonstrated in *bak1* mutants that show a delayed or almost abolished response to flg22 (Chinchilla et al. 2007). It was hypothesised that BAK1 also regulates the function of the FLS receptor and other pathogen recognition receptors since *bak1* mutants are still functional in brassinolide binding to BRI1 (Chinchilla et al. 2007).

In other root microarrays with clubroot *BAK1* was not induced or even slightly repressed at the stage of cortical infection and later during resting spore formation (Fig. 3.9, Siemens et al. 2006; Agarwal et al. 2011). According to the same microarrays, *FLS2* was slightly repressed until roughly 10 dai and very strongly at 10 dai and 23 dai (LOG2 fold change -4,13 and -2,59). Around 6 to 8 days after the first contact of the primary zoospore with a root hair, secondary zoospores emerge and reinfect the cortex (Kageyama and Asano 2009). No microarray data is available for 6 to 8 dai but it seems likely that PTI is suppressed at this time point as well and the plant fails to detect the zoospores which could explain the time lag in activation of defence responses of the plant. Since the PTI response is rather weak the blocking of just 1 component in the priming cascade is sufficient to disturb this early plant defence mechanism (Dodds and Rathjen 2010).

In cabbage plants Aist and Williams (1971) found signs of induced defence in the primary infection in root hairs as deposition of callose. No studies are published to date that describe a similar reaction for susceptible Arabidopsis Col-0 to the clubroot infection. The deposition of callose is an ubiquitous response of the plant following pathogen challenge and the right timing of the deposition is a crucial factor in successful plant defence (Voigt 2014). By slowing down pathogen invasion the plant gains time to induce other defence mechanisms through the means of gene expression. Millet et al. (2010) showed that MAMP-triggered callose deposition in roots also requires ET signaling but is independent of SA. *MYB51*, a transcription factor that is essential for the deposition of callose (Clay et al. 2009; Millet et al. 2010) and the ET-related genes, *ETR1*, *THI2.1* and

PDF1.2 were not induced in clubroot samples in this and the 2 other microarrays suggesting that indeed callose deposition does not occur in susceptible Arabidopsis roots.

Taken together, these findings imply that PTI cannot be established successfully in Arabidopsis roots and that callose is not deposited after *P. brassicae* inoculation which results in the successful colonisation of plant roots by the pathogen. However, in this study no callose staining was carried out to gain proof for this on a physiological level. For future studies it would be beneficial to stain Arabidopsis roots in different stages of pathogen development for callose deposition.

4.2.2. Regulation of defence responses in Arabidopsis with the endophyte

Upon recognition of MAMPs by extracellular receptor-like kinases signaling occurs via MAP kinase (MAPK) cascades that leads to the activation of WRKY transcription factors and consequently to the expression of defence genes and the production of phytoalexins such as camalexin (Chisholm et al. 2006; Xu et al. 2008; Dodds and Rathjen 2010). The MAPK gene *MAPKKK18* was upregulated in endophyte and co-inoculated samples at 3 dai in the microarray. Despite its putative role as WRKY-inducer, *MAPKKK18* is involved in senescence mechanisms as recently discovered (Matsuoka et al. 2015). Around 9 dai the secondary zoospores from *P. brassicae* emerge to reinfect the cortex of the roots and the so called "cortical stage" of the clubroot pathogen begins. At 14 dai large secondary plasmodia are visible and at 20 dai resting spores of the pathogen are produced and liberated (Kobelt et al. 2000; Kageyama and Asano 2009).

During the cortical infection at 9 and 14 dai co-inoculation with *A. alternatum* strongly induced *the PATHOGENESIS RELATED PR1*-gene and the *PLANT DEFENSIN PDF1.2* (Fig. 3.11). As *PR1* is a marker for systemic acquired resistance (SAR) these results indicate that the endophyte helped establish and maintain SAR in Arabidopsis roots for several days until the formation of resting spores occurred around 20 dai. An early onset of SAR is supported by significantly higher transcript levels of 4 *PR*-genes in the co-inoculated root samples at 3 dai.

In clubroot samples the induction of *PR1* was weaker. Clubroot-infected plants contain more endogenous SA and have a higher SA methylation rate than control plants (Ludwig-Müller et al. 2015). Methylation of SA results in methyl-SA (MeSA) that functions as mobile long-distance signal in SAR in tobacco but is not required for SAR in Arabidopsis (Park et al. 2007; Attaran et al. 2009). Based on the results from *in vitro* experiments with PbBSMT, Ludwig-Müller et al. (2015) hypothesised that *P. brassicae* methylates the SA signal by secreting its own methyltransferase (PbBSMT) into the host root cells. The resulting methyl-SA is then transported in the leaves where it is mainly emitted in the atmosphere or converted back to SA (Attaran et al. 2009; Ludwig-Müller et al. 2015). Methylation of the SA-signal could explain the lower *PR1* levels observed in clubroot-infected roots. The fungus might counteract this by triggering several signaling cascades

at once that enhance the SA-signal to a level that is sufficient to establish "proper" SAR which is more efficient in fighting the disease.

The concomitant activation of the jasmonate (JA) marker gene *PDF1.2* suggests that the JA-mediated defence pathway was activated at the same time. There is an extensive cross-talk between the SA-dependent and the JA-dependent pathway and under certain conditions both pathways can be activated simultaneously and enhance the overall immune response (van Wees et al. 2000). It has been shown that *PDF1.2* gene expression is directly dependent on *COI1* expression and requires signals from the JA/ET-response pathway (Pré et al. 2008). However, in this study *COI1* and *JAR1* were not induced and this was consistent over all experiments in all treatments yet still a notable induction of *PDF1.2* was measured especially after *A. alternatum* treatment. Two reasons seem likely for this observation. Either *PDF1.2* can be upregulated independently of *COI1* and *JAR1* or the findings for this gene are erroneous. As described in section 3.3.2 the transcript levels of PDF1.2 are very low in roots compared to leaves (signal intensities on the Agilent microarray from Arabidopsis roots, Auer, 3 dai: 4,76 for control samples and 12,87 for endophyte treated samples). It is unclear if an increase in transcript level of a gene with such low amounts in roots is an indication of the physiological responses that are attributed to the activation of this gene.

It is notable that at 20 dai these JA-inducible genes were strongly suppressed in clubroot treated samples and at the same time *PR1* transcript levels were low which corresponds with the conversion of SA to MeSA at 28 dai in Arabidopsis (Ludwig-Müller et al. 2015).

Taken together, these results and the findings from the literature prove that the clubroot pathogen suppresses the dual defence strategy of the plant by impairing proper signaling of the respective compounds. The increased *PR1* transcript levels in co-inoculated samples indicate that the endophyte is at least party able to reverse this repression and enables the establishment of SAR presumably through higher levels of endogenous SA. However, measurements of endogenous SA and MeSA in *A. alternatum*-co-inoculated plants are necessary to prove this concept.

THIONIN 2.1 (*THI2.1*), another marker for the JA-mediated resistance pathway, was induced at 9 dai in clubroot-infected plants and strongly upregulated at 23 dai in clubroot-infected samples of the Arabidopsis root microarray (Siemens et al. 2006). *THI2.1* is susceptible to necrotrophic pathogens like *Fusarium oxysporum* and the overexpression of this gene increased resistance against the pathogen in suceptible Arabidopsis Col-2 (Epple et al. 1995). Holtorf et al. (1998) generated Arabidopsis overexpressor lines for viscotoxin A3, a thionin from *Viscum* plants. These overexpressors developed galls as well but had a distinctly lower infection rate than the corresponding C24 wildtype. The authors hypothesised that viscotoxin might impair the primary infection or the beginning of the cortical stage of the clubroot pathogen but is ineffective once the pathogen is

established in the plant since galls are produced (Holtorf et al. 1998).

In this study the *THI2.1* gene did not play a major role in the tripartite interaction. According to the results with semi-quantitative RT-PCR it was hypothesised that this gene might be specific for the inoculation with *P. brassicae* whereas *PDF1.2* could be specific for inoculation with *A. alternatum*. As with *PDF1.2*, the amount of *THI2.1* in roots is rather low and therefore the results obtained from this gene are questionable (see discussion on this for *PDF1.2* above). Additionally, despite the regulation on transcript level *THI2.1* is susceptible to epigenetic control as well (Alvarez-Venegas et al. 2007).

The findings of this study implicate that *A. alternatum* has a beneficial effect at the beginning of the clubroot infection by facilitating detection of the pathogen and boosting plant defences in general. While the fungus travels up into other plant organs to facilitate its own distribution *P. brassicae* induces cell divisions in the roots and alters the root structure so that the local response to *P. brassicae* is stronger determined by the pathogen than by the fungus. This might be the crucial point in this interaction: the spatial separation of both invaders several days after colonisation keeps the biochemical network of the plant constantly "busy" as it adjusts its gene expression according to the needs of the mutualist *A. alternatum* in presumably all plant organs and at the same time struggles to defend itself against *P. brassicae* in the roots. In consequence, at the later stages in this interaction (starting around 14 dai) the plant is more susceptible to clubroot as the pathogen clearly disturbs the timing of defence responses and thus establishes pathogenicity.

The mutualistic interaction of beneficial microbes and their host plants require a high degree of molecular coordination between both organisms and MAMPs are an integral part of it (Van Wees et al. 2008). Some fungi are able to activate resistance but fail to provide significant protection against the challenger (Raps and Vidal 1998). This is likely the case with *A. alternatum* as well. While the endophyte helps the plant to recognize the clubroot pathogen at first sight it fails to trigger defence responses constantly as observed by lowering transcript levels of *PR1* over time during the interaction. However, constant triggering of the plant defence is a necessary criterion for successful plant defence in the long run.

A. alternatum controls mildew pathogens to some extent but molecular data for this is lacking (Romero et al. 2003; Kasselaki et al. 2006). Therefore further molecular studies with this fungus should include foliar pathogens such as mildews or *Alternaria alternata* as well to test whether the presence of the fungus in the respective tissue affects the reduction of symptoms and to further characterise the interaction with pathogens on gene expression level.

4.2.3. The role of WRKY transcription factors in the tripartite interaction

WRKY transcription factors constitute one of the largest families of transcriptional regulators that are unique in plants (Eulgem et al. 2000; Bakshi and Oelmüller 2014). They contain the WRKY domain WRKYGQK and bind preferably to the highly conserved W box of many defence-related genes. WRKYs are induced by biotic and abiotic stresses and also have a function in senescence (Zentgraf et al. 2010; Bakshi and Oelmüller 2014). They activate SA-responsive genes and are among the earliest genes induced upon pathogen infection. From Arabidopsis 72 *WRKY* genes are known so far and several have been studied in detail for their specific function also in plant-pathogen interactions (e. g. Pieterse and Van Loon 2004; Wang et al. 2006; Loake and Grant 2007; Yang et al. 2009; Wang et al. 2014).

In this study, *WRKY46* was upregulated in root samples co-inoculated with *A. alternatum* and *P. brassicae* at 3 dai in the microarray. WRKY46 is a positive transcriptional regulator of SID2 and stimulates SA biosynthesis (van Verk et al. 2011).

The transcription factor *WRKY18* was upregulated at 3 dai in the microarray by *A. alternatum* and at 4 dai by *P. brassicae* (Agarwal et al. 2011, Fig. 3.9) which suggests an important role of this gene in the tripartite interaction. The gene did not respond to the treatment with the priming solutions (CWE and autoclaved spores of *A. alternatum*) and was downregulated after *A. alternatum* inoculation in Arabidopsis roots from agar plates at 3 dai.

Mutant analyses showed that a defect in *WRKY18* slightly decreases the susceptibility of Arabidopsis to *P. brassicae* while increasing the susceptibility to the clubroot pathogen when plants are inoculated with *A. alternatum* as well. This could mean that the endophyte utilises *WRKY18* to shape its mutualistic interaction with the plant. A defect in the gene might lead to an imbalance in the relationship of *A. alternatum* and Arabidopsis that results in a higher susceptibility as the plant struggles to defend itself against 2 invaders at once. This theory is supported by the drastically reduced biomass of co-inoculated *wrky18* mutants and shows that the plants invested more resources in defence than in growth, a trade-off for SAR (Vos et al. 2013).

The 2 WRKY18 overexpressor lines were generally less susceptible to clubroot than the wildtype and their DI after co-inoculation with *A. alternatum* at a high spore concentration of 10^7spores/ml (DI=76) was close to the cut-off DI for moderate resistant plants. However, the distance between the DI ratios of co-inoculated and non-co-inoculated plants was similar to the observations from wildtype plants (Tab. 3.2). Chen and Chen (2002) found that overexpression of WRKY18 increased the transcript levels of PR-genes and is not dependent on SA but relies on the disease resistance protein NPR1.

The discrepancy at 3 dai - downregulation in axenic cultures inoculated with *A. alternatum* in contrast to the upregulation of WRKY18 in all other experiments - could be an

effect of the cultivation conditions. On the translucent agar plates the plants likely suffer from the limited space and experience light stress as the roots are permanently exposed to light during the day. In this environment the fungus might deactivate the immune response of the plant actively to facilitate its colonisation and establish a mutualistic relationship with the host plant. Another explanation could be the time point at which the roots were harvested. On agar plates the fungal spores are applied directly on the roots in contrast to experiments on soil or sand where a certain time for migration is necessary before the fungus finds roots to colonise. The downregulation of transcript levels could therefore be a result of a shifted time frame and is not comparable to the 3 dai from the hydroponic experiments. Unfortunately, so far no microarray data are available for *A. alternatum* and the data presented here are the first molecular data and novel findings for this interaction.

Overall it seems that *WRKY18* plays a role in this tripartite interaction that needs to be studied further in order to understand its relevance.

4.2.4. Regulation of MAMP-related genes in B. napus

As within Arabidopsis, the inoculation of rapeseed with *P. brassicae* did not result in significant changes of the MAMP-related genes studied (see Fig. 3.13). This could mean that *P. brassicae* elicitors are not detected by the plant and thus no PTI is established as is the case with Arabidopsis (see discussion in section 4.2.1). However, callose deposition was found in cabbagge plants as reaction to primary infection with *P. brassicae* and it was reported that 2 plant strengtheners reinforced cellular root structures in *B. napus* and *B. rapa* and reduced the disease index (Aist and Williams 1971; Kammerich et al. 2014).

Heteroconium chaetospira, an endophyte that controls *P. brassicae* at a low pathogen pressure, induced *BnPR1* and 3 genes from the JA/ET signaling cascade (*BnSAM3*, *BnACO* and *BnOPR2*) which were not studied in this work at 14 dai in a susceptible rapeseed cultivar (Lahlali et al. 2014). The authors also found an upregulation of JA/ET-dependent genes and concluded that the activation of JA/ET-mediated defences is crucial for the resistance against clubroot in rapeseed (Lahlali et al. 2013, 2014).

In this study, *A. alternatum* induced the MAPK gene *BnMAPK4* and the transcription factor *BnWRKY33* and repressed *BnPR1* and *BnPDF1.2* when applied alone at 3 dai (Fig. 3.13). *BnMPK4* is suggested to be a crosstalk gene for the coordination of SA and JA signaling pathways by activating JA signaling and suppressing SA signaling (Wang et al. 2012). This indicated, that *A. alternatum* utilises JA-dependent gene expression but as shown does not greatly enhance the resistance of plants towards clubroot. Wang et al. (2014) found that overexpression of *BnWRKY33* induces *BnPR1* and *BnPDF1.2*. These findings do not fit to the observations here and more time points need to be studied to draw a conclusion about the resistance pathways employed by *A. alternatum* in rapeseed. As described in section 1.1.4 the resistance against clubroot differs in *Brassica* cultivars

from race to race. The reason for this could be that PAMP responses vary considerably among cultivars (Lloyd et al. 2014). It would therefore be advantageous to study this interaction with new field crops that already show some tolerance to clubroot and which will be cultivated by farmers in Germany.

In conclusion it can be said that the endophyte presumably triggered the PAMP-mediated response in the investigated rapeseed cultivar " Visby" via *BnMAPK4* and thus likely caused PTI in the roots. The downregulation of *BnPR1* in this study supports this theory. However, as in the studies with Arabidopsis the role of *BnPDF1.2* is not completly clear in the interaction of rapeseed with *A. alternatum*. Since *PDF1.2* acts downstream of the JA signal an upregulation would be expected to fit to the transcript levels of the other 4 genes.

4.3. The role of hormones in defence and growth in the tripartite interaction

The role of the brassinosteroid receptor BAK1 in regard to PTI was discussed in section 4.2.1 already. Brassinosteroids such as brassinolid can induce disease resistance in plants (Nakashita et al. 2003) and also play a role in the interaction of *P. brassicae* and Arabidopsis (Siemens et al. 2002). As demonstrated previously, the brassinosteroid receptor mutant *bri1-6* is moderately resistant to clubroot (Schuller et al. 2014) and co-inoculation with *A. alternatum* lowered the DI to 50 vs. 67 in clubroot infected plants which was the lowest DI observed in this study. In the microarray the transcript levels of *BAK1* in endophyte-inoculated plants almost reached the threshold for induction (LOG2 fold change 0,97) and it is likely that the endophyte also depends on brassinosteroid signaling to shape its relationship with the plant.

Treatment of *bri1-6* mutants with 10μM propiconazole (=brassinosteroid synthesis inhibitor) promoted the plant growth after inoculation with *A. alternatum* and the biomass of *bri1-6* was larger than in the untreated wildtype which suggests that brassinosteroids might not be of major importance in the growth promotion. However, the growth promotion experiments with *bri1-6* and propiconazole were carried out once and should be repeated with different concentrations of the hormone inhibitor to verify these results.

A key enzyme in ABA synthesis, *NCED3*, was upregulated in the microarray in the co-inoculated roots at 3 dai. ABA is known as major mediator of abiotic stress responses but apparently it also plays a role in plant immunity by influencing the SA-mediated responses to biotrophic pathogens such as *Pseudomonas syringae* (Cao et al. 2011).

The impact of auxin on the clubroot infection has been extensively studied (e.g. De Vos et al. 2005; Devos et al. 2006; Ludwig-Müller 2009; Ludwig-Müller et al. 2009). Auxin is a key enzyme of the plant in the balance of mutualistic relationships that oscillate between development and defence (Ludwig-Müller 2015). Clubroot infected plants produce high

levels of auxin at the end of the secondary infection and studies showed that decreasing the auxin levels in roots leads to smaller galls but at the same time dwarfs above ground plant parts (Grsic-Rausch et al. 2000; Neuhaus et al. 2000; Ludwig-Müller et al. 2009).

In the microarray an upregulation of several *SAUR*-(*SMALL AUXIN UPREGULATED RNA*) genes was observed for all treatments. Some SAUR-family proteins can promote cell expansion presumably through modulation of auxin transport (Spartz et al. 2012). Additionally the plant expressed higher levels of several xyloglucan endotransglucosylase/hydrolase genes in all treatments which lead to cell wall loosening and cell expansion upon elevated auxin levels (De Vos et al. 2005). Both pathogens likely profit from the cell wall loosening that is required for cell expansion as it eases their way into the plant. For roots inoculated with *P. brassicae* and *A. alternatum* as well as in clubroot-infected-only roots an upregulation of *EXPANSIN A18* was found in the microarray at 3 dai (LOG2 fold change 1,27 and 1,15; see Appendix) which supports this theory.

SAUR genes might also be activated by the endophyte for the modulating of auxin transport as shown in the treatment with the auxin transport inhibitor NPA. Several beneficial microbes directly influence the distribution and levels of auxin, for example *Piriformospora indica*, a root-growth promoting endophyte, activates auxin-related genes in Chinese cabbage but not in Arabidopsis (Lee et al. 2011).

Members of the GH3 protein family conjugate auxin and other plant hormones and directly influence the susceptibility of plants to a pathogen as low levels of active auxin - and a higher amount of auxin conjugates - makes plants more resistant, a trade-off for proper growth and development (Ludwig-Müller 2015). In this study, *GH3.1* was induced in the co-inoculated samples which could be interpreted as sign for an enhanced resistance in this treatment. The same gene was upregulated in the Arabidopsis root microarray from Siemens et al. (2006) at the early timepoint 10 dai (LOG2 fold change 1,02).

GH3.5, which was strongly induced at 23 dai (LOG2 fold change 2,47; Siemens et al. 2006), did not show any regulation in this tripartite interaction at any time point. Since qPCR data for this gene is missing from 20 dai it cannot be excluded that a differential regulation occured at this time point, the sqPCR data from 20 dai did not hint on any regulation though.

In clubroot inoculated samples the *URIDINE DIPHOSPHATE GLYCOSYLTRANSFERASE 74E2* (*UGT74E2*) was upregulated. Park et al. (2011) demonstrated by the use of T-DNA insertion mutants that the suppression of *UGT74E2* increases the resistance to *Pseudomonas syringae*. They concluded from their study that SAR was enhanced in the *ugt74e2* mutant and that glycosylation of hormones such as auxin could play a role in SAR. Glycosylation changes the properties (such as the bioactivity) of the molecular acceptor. In the recent finding of a novel auxin glycosyltransferase (UGT74D1) the authors demonstrated that overexpression of *UGT74D1* affects the level of active auxins in the plant (Jin et al. 2013). As discussed above the level of active auxin in the plant also determines the

resistance to pathogens.

An upregulation of *UGT74E2* early in the interaction by *P. brassicae* indicates that the plants are more susceptible to the clubroot pathogen. This theory is supported by the upregulation of a homologue of this gene (*BN23990*) in susceptible *B. napus* stems upon infection with *Sclerotinia sclerotiorum* (Zhao et al. 2009).

Gibberellins (GAs) are likely involved in the significant stem length promotion and the early initiation of flowering that was observed after inoculation with *A. alternatum* (Auer and Ludwig-Müller 2014). The fungus induced 2 GA-related genes at 3 dai: the *GA-STIMULATED ARABIDOPSIS 6* gene (LOG2 fold change 1,62; see Appendix) in endophyte samples and the *GIBBERELLIN 20-OXIDASE 5* gene (LOG2 fold change 1,26; see Appendix), an enzyme that catalyses one of the final steps in the GA biosynthesis, in co-inoculated samples. In addition the fungus might produce gibberellins itself like *Gibberella fujikuroi* (Kawaide 2006) but this was not tested in this study. To assess the role of GAs in the interaction between the host and the fungus measurements of endogenous GA from inoculated and control plants could be carried out in *B. napus* as it was shown that GAs regulate the onset of flowering and stem elongation (Rood et al. 1989).

Additionally, rapeseed plants could be treated with a GA biosynthesis inhibitor such as chlorcholine chlorid (CCC) and the stem lengths and flower initiation determined for inoculated and control plants. Preliminary experiments with this inhibitor and Arabidopsis on agar plates did not result in any obvious growth effects as the fungus was able to promote growth on CCC-treated plants as well which might have been a result of too low CCC concentrations (data not shown).

A. alternatum induced the *ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 14* (*ERF14*) in Arabidopsis roots at 3 dai when administered alone. *ERF*-genes are positive regulators of the JA/ET-mediated defence pathways and *ERF14* specifically is crucial for the induction of ET-inducible resistance genes (Oñate-Sánchez et al. 2007; Camehl and Oelmüller 2010). In the *Piriformospora indica*-Arabidopsis interaction *ERF14* was upregulated at 7 dai in roots and required for the growth promotion as shown by a diminished growth promotion in homozygote knock-out lines for *ERF14*. Inactivation of the gene activated the expression of *PR*-genes and it was hypothesised that *ERF14* can repress *PR1* expression and therefore contributes to the establishment of the beneficial fungus *P. indica* in the roots of Arabidopsis (Camehl and Oelmüller 2010).

Three ACC synthase (ACS) genes that encode enzymes involved in ET biosynthesis were upregulated in the microarray at 3 dai: *ACS7 and ACS8* in co-inoculated and *ACS11* in clubroot-only-infected root samples. A high ACS activity is connected with an increased ET formation as these enzymes show a low abundance in normal tissues and ACS enzymes are the rate-limiting enzymes in ET biosynthesis (Li et al. 2012). All 3 *ACS* genes from this study are not regulated by the ubiquitous MPK3/MPK6 cascade that induces the expression of many defence and stress related genes and the signaling pathways which

activate the expression of *ACS* genes are not known (Li et al. 2012). The transcription factor *ORA59* was downregulated in the co-inoculated roots at 3 dai. *ORA59* integrates JA and ET signaling during plant defence and was reported to be necessary for the activation of *PDF1.2* in 14-day old Arabidopsis seedlings treated with JA and the ET-releaser ethephon (Pré et al. 2008; Pieterse et al. 2012). This finding is contradictionary to elevated *PDF1.2* levels in the microarray (S. Auer), however, as discussed in section 4.2.2 the elevated *PDF1.2* levels might not be of importance.

4.4. Implications regarding the production of defence compounds in Arabidopsis

MYB51, a transcription factor that is essential for the deposition of callose and is a positive regulator of indole glucosinolate (IGS) biosynthesis (Clay et al. 2009; Millet et al. 2010), was not induced in clubroot samples in this and other microarrays (Siemens et al. 2006; Agarwal et al. 2011). A low signal was detected in co-inoculated plants (LOG2 fold change 0,20) which was slightly higher when the endophyte was administered alone (0,39). In clubroot-infected plants the transcript levels of *MYB51* were lower than in the controls but did not reach the threshold of -1 (LOG2 fold change -0,31).

MYB51 activates the promotors of several genes involved in IGS biosynthesis (Gigolashvili et al. 2007) and the lowered transcript level in clubroot samples at 3 dai might affect IGS biosynthesis negatively. By the analysis of *myb51* and *cyp71a13* mutants, Schlaeppi and Mauch (2010) found that disease resistance in Arabidopsis against *Phytopththora brassicae* relies on a combination of IGS and the phytoalexin camalexin. In their study IGS were crucial for the resistance to penetration by the pathogen and later helped induce camalexin-mediated defence.

Several studies have analysed glucosinolate (GSL) contents in root galls or at earlier stages of the pathogen development (reviewed in Ludwig-Müller 2009). Though the results varied over species and experiments conducted it was hypothesised that an increase in aliphatic and aromatic GSL in root galls might be a defence response of the host the clubroot pathogen is well adapted to. In the microarray from Siemens et al. (2006) genes involved in aliphatic GLS synthesis were upregulated at the early time point and downregulated at the later time point during gall formation which indicates that *P. brassicae* represses the production of toxic compounds during gall formation (Ludwig-Müller 2009).

In this study the P450 monooxygenase *CYP71A12* (*AT2G30750*) was studied, a gene very similar to *CYP71A13* (*AT2G30770*), which catalyses the first step in camalexin biosynthesis and is considered as a major antibiotic of Arabidopsis (Nafisi et al. 2007; Ludwig-Müller et al. 2009; Schlaeppi and Mauch 2010; Millet et al. 2010). *CYP71A12/13* was not regulated at 3 dai in roots co-inoculated with both microorganisms and the tendency at other time points was rather a repression than an induction but in root galls *CYP71A12/13*

was upregulated (Agarwal et al. 2011; Siemens et al. 2006). The phytoalexin camalexin and GLS both share a precursor in their biosynthetic pathways.

The *EPITHIOSPECIFIER PROTEIN*-gene *ESP* (*AT1G54040*), was upregulated specifically in co-inoculated roots at 3 dai in the microarray. Together with myrosinase ESP proteins degrade GLS to toxic (epi)thiocyanates. However *P. brassicae* seems to hijack this by increasing the levels of nitrilase which leads to higher auxin levels and gall formation (Diederichsen et al. 2014). However, *ESP* might trigger defence mechanisms since it interacts with WRKY53 (Miao and Zentgraf 2007).

4.5. Novel findings for the endophytic fungus A. alternatum

With microscopy it could be shown that the fungus grows readily in and around the roots of Arabidopsis presumably in the intercellular space. PCR confirmed that the fungus does not only stay in the roots but travels up to the leaves as well within a few days. Unfortunately the exact location of *A. alternatum* within the plant could not be determined with light and scanning electron microscopy, a problem that has been reported for *Acremonium* species before (Grunewaldt-Stöcker et al. 2007). A fluorescence protein-marked strain would have been desirable in this case to detect the exact position of fungal hyphae in the plant organs. Unfortunately such a strain is not available for *A. alternatum* yet. The sister species *A. strictum* has been successfully transformed with GFP (Grunewaldt-Stöcker et al. 2007) so it might be possible to do the same in *A. alternatum* in the future. However, the main focus of this work was on the study of resistance genes in Arabidopsis.

4.5.1. Competition over resources - the complex tripartite interaction between Plasmodiophora, Acremonium and Arabidopsis

In most of the experiments clubroot-infected plants benefitted from a simultaneous inoculation with *A. alternatum* and produced more biomass and lived longer than clubroot-infected plants without the fungal inoculation. To balance the mutualistic relationship with the plant the fungus employs several signaling pathways and causes a mild induction of resistance. *A. alternatum* and *P. brassicae* likely compete over carbon sources of the plant.

The plant leaches carbons such as glucose, fructose and sucrose and these plant-derived sugars are major contributors to the successful colonisation of microbes in the roots (Sun et al. 2014). The clubroot pathogen *P. brassicae* establishes a carbon sink in the roots to facilitate its own propagation (Evans and Scholes 1995).

The endophyte can be found in leaves of infected plants as well; thus the fungus could metabolise carbon sources not only from the roots but also from leaves or upper plant parts. Raps and Vidal (1998) did not find an altered carbon-nitrogen ratio in cabbage plants

inoculated with *A. alternatum*. However, despite its ability to trigger immune responses of the plant *A. alternatum* might manipulate the carbon sink created by *P. brassicae* in order to utilise the carbon itself. That *A. alternatum* rapidly uses the sugars provided by the plant is apparent in all experiments with carbon free medium. Here the growth promotion occured as well but not to the same magnitude - in carbon free medium promotion was roughly 1 tenth of the promotion in comparison to carbon containing medium (Fig. 3.19). Further analysation of the microarray data with the focus on transport and photosynthesis related genes is necessary to better understand the role of carbon in this interaction.

A. alternatum and *P. brassicae* might also compete over sterols in the plants since for fungi the sequestration of sterols is essential for further growth (Hendrix 1970). In resting spores of *P. brassicae* sterols have been found and clubroot symptoms can be reduced with the application of sterol biosynthesis inhibitors though the latter affect plant growth negatively as well (Knights 1970; Choi et al. 2005). The recently published genome of *P. brassicae* will further increase our knowledge about the biochemical potential of this species (Schwelm et al. 2015).

4.6. The endophyte A. alternatum has exploitable biocontrol potential

Research papers from the 1980s and 1990s indicate a role for *A. alternatum* as biocontrol agent against tar spot and several mildew pathogens. In this study it was shown that the fungus can control the development of the soil-borne clubroot pathogen *P. brassicae* to some extent in Arabidopsis and 2 German varieties of rapeseed (*Brassica napus* var. Ability and var. Visby). Treatment with a high amount of a fungal cell wall extract decreased pathogen symptoms in rapeseed by 1 third which makes this fungus an interesting candidate for the integration in pest management. The fungus can solubilise calcium bound phosphate *in vitro* and has a positive influence on the growth of axenic Arabidopsis under salt stress conditions. The positive effect of the fungus in salt-stressed plants needs to be verified on soil preferably with crop plants such as cabbage species. Other experiments could involve higher temperatures and drought stress as well as biotic stresses from other phytopathogens. It would be desirable to test the effect of the fungus under abiotic stress conditions also in the context of clubroot-infected cabbage plants.

A. alternatum shifts senescence patterns by inducing early flowering and increases the yield of rapeseed which is highly desireable in the rapeseed industry. The potential of this fungus for the production of secondary metabolites was not tested in this study but it is likely that the fungus has potential here as many endophytes produce specific compounds (Schulz et al. 2002). Several novel secondary metabolites have been described from other *Acremonium* species, e. g. acremines, oxepinamides and fumiquinazolines (Belofsky et al. 2000; Assante et al. 2005).

Several plant strengtheners are on the market which utilise the beneficial effect of soil microbes. In Germany 2 products that are based on beneficial effects of *Bacillus subtilis* strains are sold: FZB24(R)WG and Contans(R)WG which is used against *Sclerotinia sclerotiorum.*

It is imaginable to develop a similar product against clubroot in the near future that contains, among other plant strenghtening substances, active cell wall parts of this fungus. For this more research is necessary to identify which fraction of the cell wall yields the biocontrol effects against clubroot.

4.7. Conclusions and outlook

This study provides novel insights in the tripartite interaction of the plant *Arabidopsis thaliana* with the endophyte *Acremonium alternatum* and the clubroot pathogen *Plasmodiophora brassicae*.

The early hypothesis that the beneficial fungus *Acremonium alternatum* triggers plant resistance genes in Arabidopsis was confirmed and it was found that this fungus can establish pathogen-triggered immunity in the plant by elevating the transcript levels of 2 crucial receptors for this first line of plant defence. Several results point to an establishment of systemic acquired resistance in plant roots until the point of resting spore production of *P. brassicae*. The final proof for the successful pathogen-triggered immunity would be a callose staining in infected tissues which was not carried out in this study.

The fungus increases the health of plants under clubroot pressure by increasing the biomass and enhancing survival rates of clubroot-infected plants.

Both of the tested German rapeseed cultivars are susceptible to clubroot though it seemed that the winter variety "Visby" is less susceptible to the disease. Visby has been cultivated for several years on soils in Saxony and showed constantly good yields. However, as each year new rapeseed varieties with increased seed yields reach the market and more farmers shift to hybrid seeds, Visby will likely be taken from the market soon.

Pre-treatment of rapeseed with inactivated spores and a crude cell wall extract of *A. alternatum* successfully lowered the clubroot disease symptoms. The best result was achieved with a high amount (4%) of crude cell wall extract from the fungus which reduced the symptoms of the highly susceptible *B. napus* var. Ability by 28%.

The fungus showed an ability to solubilise calcium phosphate but did not produce antagonistic enzymes such as cellulase, chitinase or protease *in vitro*. It also did not show a huge competitive potential against generalistic phytopathogens such as *Aspergillus niger* or several *Fusarium* species.

Preliminary experiments give rise to the theory that the fungus can reduce the abiotic stress of plants and makes them more competitive by increasing stem lengths and thus

minimising shading effects.

Overall, this fungus is a very good candidate for future applications in integrative pest management. It is easy to cultivate, sporulates readily and can colonise monocyts and dicots. The application of a cell wall extract has a positive effect on clubroot-infected plants and could be used in larger scales as plant strengthener against phytopathogens.

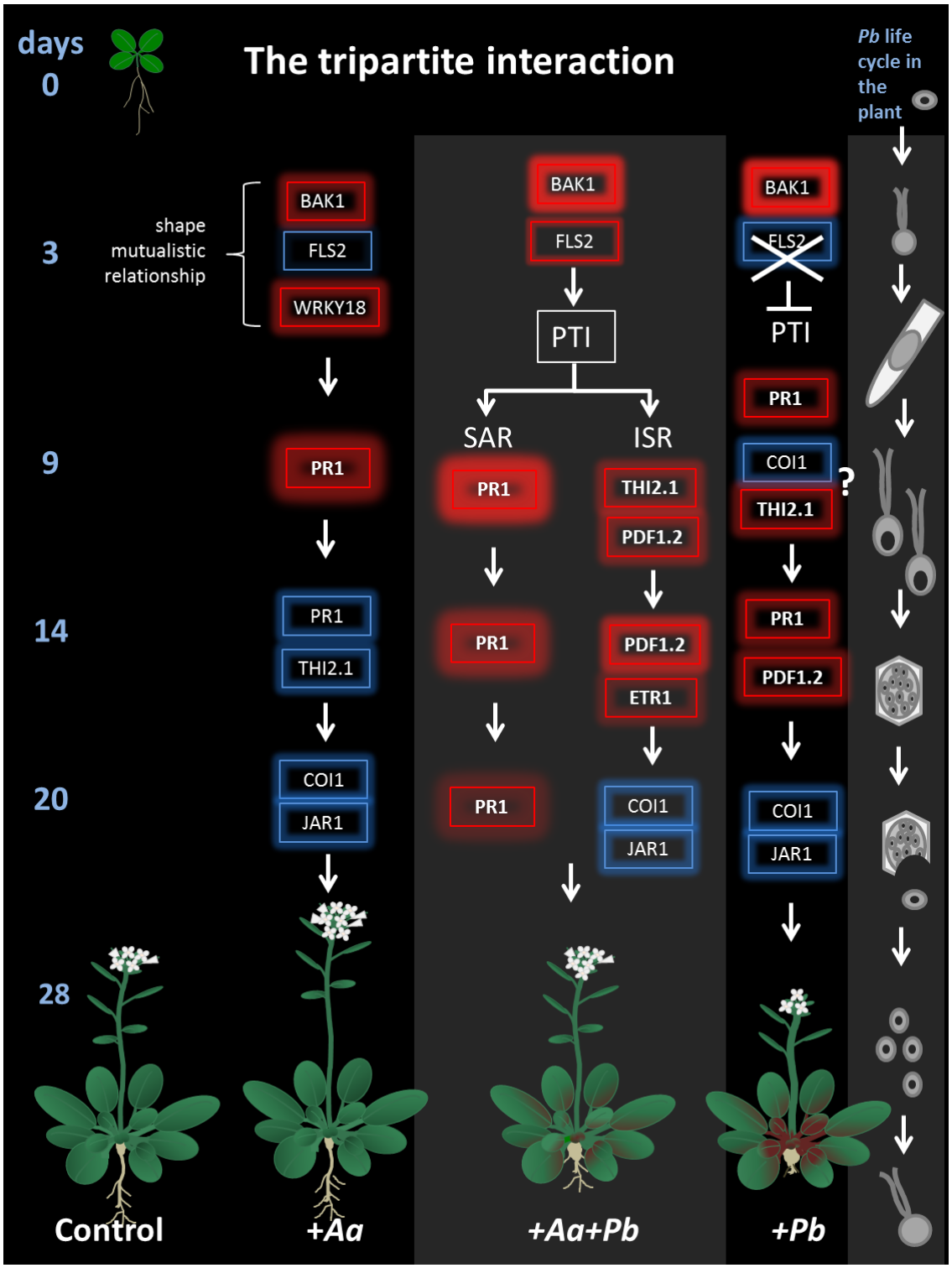


Figure 4.1.: This graphic overview of the tripartite interaction highlights the major findings of this study: The endophyte *Acremonium alternatum* establishes a mutualistic relationship with Arabidopsis. Upon challenge with the clubroot pathogen *Plasmodiophora brassicae* it triggers immune responses of the plant and activates the 2 major defence pathways SAR and ISR simultaneously. The plant benefits from this interaction and develops smaller root galls, produces more biomass, develops more flowers and increases stem length. However, full control of the clubroot pathogen is not achieved with *A. alternatum* and open questions remain regarding the fine-tuning of plant defence in this tripartite interaction. Abbreviations: *Aa* - *Acremonium alternatum*, *Pb* - *Plasmodiophora brassicae*, PTI - PAMP triggered immunity, ISR - induced systemic resistance, SAR - systemic acquired resistance. Colours of the gene boxes indicate upregulation (red) or downregulation of the respective genes.

A. Appendix

A.1. Microarray data

Table A.1.: Microarray data of Arabidopsis roots treated with *P. brassicae* spore solution (2×10^7 spores/ml) and harvested 3 days after infection. Gene-ID represents genes according to annotation of The Arabidopsis Infromation resource (TAIR), LOG2 values indicate up- ($\geq$1,0) or downregulation ($\leq$1,0). Description of genes according to TAIR

Gene-ID	log2(P vs C)	P-value	description
AT1G68520.1	-2,600	0,034	B-box type zinc finger protein with CCT domain
AT4G10250.1	-2,573	0,014	HSP20-like chaperones superfamily protein
AT3G22142.1	-2,527	0,000	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT4G34035.1	-1,735	0,046	pre-tRNA
AT5G36000.1	-1,643	0,001	BEST Arabidopsis thaliana protein match is: reduced male fertility (TAIR:AT3G61730.1)
AT5G18320.1	-1,599	0,001	ARM repeat superfamily protein
AT1G33200.1	-1,495	0,033	transposable element gene
AT3G23080.2	-1,468	0,014	Polyketide cyclase/dehydrase and lipid transport superfamily protein
AT3G10405.1	-1,445	0,012	unknown protein
AT5G23260.2	-1,346	0,020	K-box region and MADS-box transcription factor family protein
AT5G53048.1	-1,340	0,008	other RNA
AT1G50180.1	-1,331	0,003	NB-ARC domain-containing disease resistance protein
AT5G27603.1	-1,324	0,018	transposable element gene
AT5G07310.1	-1,312	0,001	Integrase-type DNA-binding superfamily protein
AT5G61950.1	-1,292	0,000	Ubiquitin carboxyl-terminal hydrolase-related protein
AT4G10060.1	-1,288	0,046	Beta-glucosidase
AT1G61275.1	-1,275	0,018	U12
AT3G46230.1	-1,274	0,025	heat shock protein 17.4
AT5G65410.1	-1,273	0,030	homeobox protein 25
AT3G06900.1	-1,262	0,024	U4.2
AT1G53540.1	-1,259	0,033	HSP20-like chaperones superfamily protein

Gene-ID	log2(P vs C)	P-value	description
AT1G63430.2	-1,226	0,025	Leucine-rich repeat protein kinase family protein
AT1G05680.1	-1,219	0,010	Uridine diphosphate glycosyltransferase 74E2
AT5G38200.1	-1,200	0,004	Class I glutamine amidotransferase-like superfamily protein
AT1G52500.1	-1,197	0,025	MUTM homolog-1
AT3G59400.1	-1,193	0,049	enzyme binding
AT2G36110.1	-1,189	0,036	Polynucleotidyl transferase
AT2G15530.4	-1,159	0,027	RING/U-box superfamily protein
AT1G78930.1	-1,151	0,008	Mitochondrial transcription termination factor family protein
AT3G45950.1	-1,147	0,029	Pre-mRNA splicing Prp18-interacting factor
AT2G35260.1	-1,145	0,004	unknown protein
AT3G54440.3	-1,142	0,012	glycoside hydrolase family 2 protein
AT5G52800.1	-1,134	0,031	DNA primases
AT1G70430.1	-1,097	0,013	Protein kinase superfamily protein
AT5G55270.1	-1,094	0,001	Protein of unknown function (DUF295)
AT1G05061.1	-1,094	0,032	Pseudogene of AT2G32630
AT5G42800.1	-1,093	0,010	dihydroflavonol 4-reductase
AT4G23890.1	-1,091	0,002	unknown protein
AT3G54410.1	-1,089	0,022	Protein of unknown function (DUF1163)
AT1G13800.1	-1,038	0,010	Tetratricopeptide repeat (TPR)-like superfamily protein
AT3G28945.1	-1,036	0,044	transposable element gene
AT4G02770.1	-1,030	0,023	photosystem I subunit D-1
AT3G01316.1	-1,024	0,045	snoRNA
AT1G64800.1	-1,024	0,019	DNA binding
AT3G52260.3	-1,022	0,025	Pseudouridine synthase family protein
AT3G47800.1	-1,006	0,013	Galactose mutarotase-like superfamily protein
AT1G65200.1	-1,001	0,003	Ubiquitin carboxyl-terminal hydrolase-related protein
AT1G10980.1	1,001	0,031	Lung seven transmembrane receptor family protein
AT5G04960.1	1,002	0,031	Plant invertase/pectin methylesterase inhibitor superfamily
AT5G44500.2	1,006	0,003	Small nuclear ribonucleoprotein family protein
AT2G28860.1	1,008	0,046	cytochrome P450
AT1G16515.1	1,011	0,011	unknown protein
AT1G67856.1	1,013	0,006	RING/U-box superfamily protein
AT1G61630.1	1,014	0,039	equilibrative nucleoside transporter 7
AT5G24105.1	1,015	0,044	arabinogalactan protein 41
AT1G73580.1	1,015	0,013	Calcium-dependent lipid-binding (CaLB domain) family protein
AT5G26930.1	1,016	0,007	GATA transcription factor 23

Gene-ID	log2(P vs C)	P-value	description
AT5G64850.1	1,019	0,045	FUNCTIONS IN: molecular_function unknown
AT1G57835.1	1,020	0,043	unknown gene
AT5G23030.1	1,031	0,004	tetraspanin12
AT5G24313.1	1,034	0,036	unknown protein
AT5G12270.1	1,040	0,020	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AT5G13170.1	1,043	0,029	senescence-associated gene 29
AT1G72520.1	1,044	0,038	PLAT/LH2 domain-containing lipoxygenase family protein
AT2G20453.1	1,048	0,022	unknown protein
AT1G47410.2	1,049	0,010	unknown protein
AT3G60280.1	1,055	0,041	uclacyanin 3
AT1G09950.1	1,057	0,004	RESPONSE TO ABA AND SALT 1
AT5G24180.1	1,062	0,008	Lipase class 3-related protein
AT1G77525.1	1,068	0,022	unknown protein
AT5G35525.1	1,068	0,037	PLAC8 family protein
AT5G63250.1	1,083	0,001	Carbohydrate-binding X8 domain superfamily protein
AT3G26510.5	1,083	0,008	Octicosapeptide/Phox/Bem1p family protein
AT1G12940.1	1,091	0,013	nitrate transporter2.5
AT5G63270.1	1,093	0,037	RPM1-interacting protein 4 (RIN4) family protein
AT5G57530.1	1,097	0,017	xyloglucan endotransglucosylase/hydrolase 12
AT5G47980.1	1,097	0,049	HXXXD-type acyl-transferase family protein
AT5G38895.1	1,099	0,034	RING/U-box superfamily protein
AT3G17185.2	1,101	0,024	TAS3/TASIR-ARF (TRANS-ACTING SIRNA3)
AT5G40430.1	1,104	0,048	myb domain protein 22
AT5G22390.1	1,105	0,046	Protein of unknown function (DUF3049)
AT4G11780.1	1,110	0,020	unknown protein
AT4G11020.1	1,114	0,014	unknown protein
AT1G60190.1	1,115	0,009	ARM repeat superfamily protein
AT5G43650.1	1,124	0,005	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT5G39480.1	1,130	0,023	F-box family protein
AT1G72430.1	1,135	0,010	SAUR-like auxin-responsive protein family
AT5G10500.1	1,141	0,000	Kinase interacting (KIP1-like) family protein
AT3G01730.1	1,142	0,015	unknown protein
AT1G34330.1	1,145	0,044	pseudogene
AT1G62980.1	1,148	0,002	expansin A18
AT3G51970.1	1,148	0,037	acyl-CoA sterol acyl transferase 1
AT5G49730.1	1,149	0,004	ferric reduction oxidase 6
AT5G35510.1	1,151	0,003	unknown protein

Gene-ID	log2(P vs C)	P-value	description
AT1G33070.1	1,154	0,048	MADS-box family protein
AT3G22830.1	1,154	0,037	heat shock transcription factor A6B
AT1G70985.1	1,162	0,005	hydroxyproline-rich glycoprotein family protein
AT1G20310.1	1,162	0,020	unknown protein
AT3G50580.1	1,175	0,038	LOCATED IN: endomembrane system
AT4G15755.1	1,181	0,031	Calcium-dependent lipid-binding (CaLB domain) family protein
AT5G05365.1	1,187	0,026	Heavy metal transport/detoxification superfamily protein
AT1G22220.1	1,188	0,046	F-box family protein
AT5G36140.1	1,193	0,000	cytochrome P450
AT1G35140.1	1,196	0,041	Phosphate-responsive 1 family protein
AT5G19790.1	1,205	0,008	related to AP2 11
AT4G20190.1	1,208	0,006	unknown protein
AT2G18800.1	1,233	0,008	xyloglucan endotransglucosylase/hydrolase 21
AT3G10710.1	1,242	0,004	root hair specific 12
AT5G22240.1	1,266	0,002	Ovate family protein
AT4G30320.1	1,266	0,003	CAP (Cysteine-rich secretory proteins
AT5G28800.1	1,306	0,006	unknown protein
AT4G34419.1	1,332	0,002	unknown protein
AT1G74430.1	1,343	0,001	myb domain protein 95
AT5G51990.1	1,354	0,047	C-repeat-binding factor 4
AT1G02575.1	1,355	0,007	unknown protein
AT5G21130.1	1,358	0,030	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AT1G50930.1	1,360	0,034	unknown protein
AT5G20935.1	1,363	0,001	unknown protein
AT5G52547.1	1,372	0,039	BEST Arabidopsis thaliana protein match is: DNA binding (TAIR:AT3G47680.1)
AT1G30560.1	1,374	0,047	Major facilitator superfamily protein
AT5G45650.1	1,399	0,003	subtilase family protein
AT2G28850.1	1,421	0,026	cytochrome P450
AT5G22150.1	1,432	0,026	unknown protein
AT5G46417.1	1,442	0,021	unknown protein
AT4G21192.1	1,451	0,012	Cytochrome c oxidase biogenesis protein Cmc1-like
AT3G32896.1	1,477	0,024	unknown protein
AT3G11940.1	1,485	0,009	ribosomal protein 5A
AT4G35165.1	1,487	0,014	Protein of unknown function (DUF1278)
AT5G52145.1	1,514	0,008	Encodes a defensin-like (DEFL) family protein.
AT5G41530.1	1,534	0,012	BEST Arabidopsis thaliana protein match is: DNA-binding storekeeper protein-related (TAIR:AT5G14280.1)

Gene-ID	log2(P vs C)	P-value	description
AT1G75717.1	1,541	0,020	unknown protein
AT1G73325.1	1,542	0,021	Kunitz family trypsin and protease inhibitor protein
AT1G11655.1	1,543	0,043	unknown protein
AT5G36270.1	1,548	0,003	pseudogene of dehydroascorbate reductase
AT3G07490.1	1,555	0,003	ARF-GAP domain 11
AT4G22630.1	1,584	0,009	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT4G27930.1	1,627	0,028	unknown protein
AT4G08040.1	1,689	0,007	1-aminocyclopropane-1-carboxylate synthase 11
AT5G52020.1	1,805	0,004	Integrase-type DNA-binding superfamily protein
AT2G03822.1	2,102	0,025	unknown protein
AT5G36130.1	2,233	0,010	Cytochrome P450 superfamily protein
AT1G24580.1	2,246	0,022	RING/U-box superfamily protein
AT5G23903.1	2,421	0,003	unknown protein

Table A.2.: Microarray data of Arabidopsis roots treated with *A. alternatum* and *P. brassicae* solutions (2×10^7 spores/ml) and harvested 3 days after infection. Gene-ID represents genes according to annotation of The Arabidopsis Infromation resource (TAIR), LOG2 values indicate up- ($\geq$1,0) or downregulation ($\leq$1,0). Description of genes according to TAIR.

Gene-ID	log2(AP vs C)	P-value	description
AT1G78450.1	-2,305	0,027	SOUL heme-binding family protein
AT1G31580.1	-2,131	0,021	ECS1
AT4G34035.1	-2,000	0,032	pre-tRNA
AT5G27603.1	-1,768	0,007	transposable element gene
AT5G57180.3	-1,713	0,019	chloroplast import apparatus 2
AT2G42280.2	-1,713	0,041	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT4G01430.1	-1,600	0,044	nodulin MtN21 /EamA-like transporter family protein
AT4G13300.1	-1,597	0,036	terpenoid synthase 13
AT3G46190.1	-1,519	0,000	TRAF-like family protein
AT1G06160.1	-1,466	0,000	octadecanoid-responsive Arabidopsis AP2/ERF 59
AT1G25440.1	-1,460	0,045	B-box type zinc finger protein with CCT domain
AT3G06900.1	-1,445	0,018	U4.2
AT5G23260.2	-1,382	0,019	K-box region and MADS-box transcription factor family protein
AT1G61275.1	-1,322	0,014	U12
AT3G56860.3	-1,315	0,001	UBP1-associated protein 2A
AT5G22280.3	-1,258	0,015	unknown protein

Gene-ID	log2(AP vs C)	P-value	description
AT3G22142.1	-1,253	0,005	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT5G61940.1	-1,248	0,004	Ubiquitin carboxyl-terminal hydrolase-related protein
AT1G29600.1	-1,242	0,015	Zinc finger C-x8-C-x5-C-x3-H type family protein
AT2G42370.1	-1,240	0,000	unknown protein
AT5G54570.1	-1,237	0,005	beta glucosidase 41
AT4G35410.1	-1,190	0,016	Clathrin adaptor complex small chain family protein
AT3G28945.1	-1,185	0,011	transposable element gene
AT3G59950.2	-1,181	0,044	Peptidase family C54 protein
AT3G01316.1	-1,177	0,039	snoRNA
AT2G25090.1	-1,172	0,010	CBL-interacting protein kinase 16
AT5G45020.2	-1,168	0,004	Glutathione S-transferase family protein
AT1G02230.1	-1,165	0,046	NAC domain containing protein 4
AT1G49200.1	-1,154	0,028	RING/U-box superfamily protein
AT2G45950.2	-1,149	0,007	SKP1-like 20
AT3G54410.1	-1,145	0,039	Protein of unknown function (DUF1163)
AT4G14905.2	-1,141	0,023	Galactose oxidase/kelch repeat superfamily protein
AT2G15530.4	-1,140	0,031	RING/U-box superfamily protein
AT1G50180.1	-1,121	0,005	NB-ARC domain-containing disease resistance protein
AT1G08115.1	-1,114	0,025	snRNA
AT1G37030.1	-1,102	0,009	transposable element gene
AT5G55740.1	-1,094	0,024	Tetratricopeptide repeat (TPR)-like superfamily protein
AT5G52800.1	-1,086	0,020	DNA primases
AT3G28925.1	-1,083	0,045	BEST Arabidopsis thaliana protein match is: structural maintenance of chromosome 3 (TAIR:AT5G48600.2)
AT2G19280.1	-1,076	0,011	Pentatricopeptide repeat (PPR) superfamily protein
AT1G63430.2	-1,072	0,029	Leucine-rich repeat protein kinase family protein
AT1G21270.1	-1,068	0,033	wall-associated kinase 2
AT3G45290.1	-1,063	0,014	Seven transmembrane MLO family protein
AT1G70620.2	-1,051	0,001	cyclin-related
AT5G38980.1	-1,049	0,047	unknown protein
AT1G24148.1	-1,048	0,017	unknown protein
AT5G04050.1	-1,029	0,038	RNA-directed DNA polymerase (reverse transcriptase)
AT1G54080.1	-1,027	0,032	oligouridylate-binding protein 1A
AT5G15830.1	-1,021	0,030	basic leucine-zipper 3
AT2G02795.1	-1,018	0,004	unknown protein
AT3G14185.1	-1,007	0,012	other RNA

Gene-ID	log2(AP vs C)	P-value	description
AT4G09010.1	-1,005	0,025	ascorbate peroxidase 4
AT1G31050.1	-1,003	0,047	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT3G22275.1	1,002	0,020	unknown protein
AT5G10380.1	1,004	0,000	RING/U-box superfamily protein
AT4G18180.1	1,007	0,032	Pectin lyase-like superfamily protein
AT4G25390.1	1,007	0,000	Protein kinase superfamily protein
AT4G26200.1	1,007	0,000	1-amino-cyclopropane-1-carboxylate synthase 7
AT2G32210.1	1,008	0,000	unknown protein
AT2G41010.1	1,015	0,012	calmodulin (CAM)-binding protein of 25 kDa
AT3G14870.2	1,020	0,003	Plant protein of unknown function (DUF641)
AT2G14960.1	1,021	0,006	Auxin-responsive GH3 family protein
AT3G16510.1	1,023	0,039	Calcium-dependent lipid-binding (CaLB domain) family protein
AT5G54165.1	1,027	0,016	unknown protein
AT5G57010.1	1,027	0,038	calmodulin-binding family protein
AT1G05540.1	1,027	0,042	Protein of unknown function (DUF295)
AT2G17230.1	1,030	0,014	EXORDIUM like 5
AT5G06320.1	1,031	0,017	NDR1/HIN1-like 3
AT5G61890.1	1,033	0,005	Integrase-type DNA-binding superfamily protein
AT5G01750.1	1,034	0,035	Protein of unknown function (DUF567)
AT4G21192.1	1,035	0,031	Cytochrome c oxidase biogenesis protein Cmc1-like
AT1G10385.1	1,037	0,006	Vps51/Vps67 family (components of vesicular transport) protein
AT1G15010.1	1,043	0,000	unknown protein
AT2G20562.1	1,047	0,017	unknown protein
AT1G05000.2	1,047	0,019	Phosphotyrosine protein phosphatases superfamily protein
AT5G01100.1	1,047	0,015	O-fucosyltransferase family protein
AT4G25433.1	1,049	0,040	peptidoglycan-binding LysM domain-containing protein
AT3G25600.1	1,049	0,006	Calcium-binding EF-hand family protein
AT1G55330.1	1,050	0,002	arabinogalactan protein 21
AT1G76240.1	1,053	0,037	Arabidopsis protein of unknown function (DUF241)
AT2G16630.1	1,054	0,022	Pollen Ole e 1 allergen and extensin family protein
AT4G33985.1	1,060	0,016	Protein of unknown function (DUF1685)
AT1G48267.1	1,060	0,034	MIR161
AT5G10750.1	1,063	0,003	Protein of unknown function (DUF1336)
AT1G22470.1	1,071	0,009	unknown protein
AT3G06890.1	1,072	0,008	unknown protein
AT5G13170.1	1,072	0,040	senescence-associated gene 29

Gene-ID	log2(AP vs C)	P-value	description
AT4G35320.1	1,073	0,036	unknown protein
AT1G08860.1	1,073	0,002	Calcium-dependent phospholipid-binding Copine family protein
AT3G03280.1	1,075	0,001	unknown protein
AT4G39670.1	1,083	0,013	Glycolipid transfer protein (GLTP) family protein
AT1G43800.1	1,084	0,002	Plant stearoyl-acyl-carrier-protein desaturase family protein
AT1G23130.1	1,086	0,022	Polyketide cyclase/dehydrase and lipid transport superfamily protein
AT1G70090.1	1,088	0,004	glucosyl transferase family 8
AT3G51970.1	1,094	0,019	acyl-CoA sterol acyl transferase 1
AT1G29540.1	1,099	0,010	unknown protein
AT4G37360.1	1,099	0,002	cytochrome P450
AT1G53060.1	1,102	0,010	Legume lectin family protein
AT5G11160.1	1,103	0,010	adenine phosphoribosyltransferase 5
AT4G30440.1	1,104	0,010	UDP-D-glucuronate 4-epimerase 1
AT3G12410.1	1,105	0,048	Polynucleotidyl transferase
AT1G72520.1	1,105	0,022	PLAT/LH2 domain-containing lipoxygenase family protein
AT3G55646.1	1,106	0,008	unknown protein
AT2G17740.1	1,106	0,044	Cysteine/Histidine-rich C1 domain family protein
AT1G18290.1	1,108	0,000	unknown protein
AT2G41410.1	1,110	0,001	Calcium-binding EF-hand family protein
AT5G13700.1	1,110	0,006	polyamine oxidase 1
AT3G25240.1	1,111	0,017	Protein of unknown function (DUF506)
AT1G51030.1	1,112	0,043	unknown protein
AT1G61810.3	1,115	0,007	beta-glucosidase 45
AT5G66320.1	1,115	0,042	GATA transcription factor 5
AT5G64450.1	1,120	0,008	BEST Arabidopsis thaliana protein match is: Putative endonuclease or glycosyl hydrolase (TAIR:AT3G62200.1)
AT2G37870.1	1,121	0,042	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT2G38340.1	1,122	0,002	Integrase-type DNA-binding superfamily protein
AT1G22810.1	1,127	0,005	Integrase-type DNA-binding superfamily protein
AT5G41750.2	1,127	0,001	Disease resistance protein (TIR-NBS-LRR class) family
AT4G08555.1	1,127	0,030	unknown protein
AT4G01575.1	1,130	0,010	serine protease inhibitor
AT1G57630.1	1,135	0,013	Toll-Interleukin-Resistance (TIR) domain family protein
AT2G02990.1	1,135	0,001	ribonuclease 1
AT1G07680.1	1,136	0,017	unknown protein
AT4G05018.1	1,136	0,001	unknown protein

Gene-ID	log2(AP vs C)	P-value	description
AT5G41670.1	1,154	0,004	6-phosphogluconate dehydrogenase family protein
AT1G19900.1	1,163	0,041	glyoxal oxidase-related protein
AT4G34300.1	1,165	0,000	Cupredoxin superfamily protein
AT4G13000.1	1,168	0,014	AGC (cAMP-dependent
AT4G36950.1	1,170	0,040	mitogen-activated protein kinase kinase kinase 21
AT5G64310.1	1,171	0,010	arabinogalactan protein 1
AT1G11055.1	1,173	0,016	Encodes a defensin-like (DEFL) family protein.
AT1G49150.1	1,176	0,013	unknown protein
AT5G01640.1	1,179	0,000	prenylated RAB acceptor 1.B5
AT1G53820.1	1,181	0,042	RING/U-box superfamily protein
AT5G03204.1	1,186	0,040	unknown protein
AT4G30320.1	1,186	0,031	CAP (Cysteine-rich secretory proteins
AT5G63130.1	1,192	0,050	Octicosapeptide/Phox/Bem1p family protein
AT3G10710.1	1,196	0,043	root hair specific 12
AT4G05030.1	1,200	0,014	Copper transport protein family
AT1G67100.1	1,209	0,017	LOB domain-containing protein 40
AT2G32470.1	1,218	0,014	F-box associated ubiquitination effector family protein
AT4G33930.1	1,219	0,017	Cupredoxin superfamily protein
AT1G11655.1	1,225	0,011	unknown protein
AT1G53620.1	1,225	0,002	unknown protein
AT3G14440.1	1,233	0,000	nine-cis-epoxycarotenoid dioxygenase 3
AT4G20006.1	1,237	0,002	unknown protein
AT3G46080.1	1,238	0,049	C2H2-type zinc finger family protein
AT5G64590.1	1,240	0,037	BEST Arabidopsis thaliana protein match is: Putative endonuclease or glycosyl hydrolase (TAIR:AT3G62200.1)
AT2G14247.1	1,242	0,000	Expressed protein
AT4G33430.2	1,246	0,012	BRI1-associated receptor kinase
AT1G72950.1	1,246	0,029	Disease resistance protein (TIR-NBS class)
AT5G36900.1	1,256	0,017	unknown protein
AT2G41730.1	1,260	0,000	unknown protein
AT2G22500.1	1,261	0,030	uncoupling protein 5
AT1G44090.1	1,262	0,004	gibberellin 20-oxidase 5
AT5G41900.1	1,262	0,009	alpha/beta-Hydrolases superfamily protein
AT3G27320.2	1,263	0,007	alpha/beta-Hydrolases superfamily protein
AT2G43290.1	1,264	0,003	Calcium-binding EF-hand family protein
AT4G29450.1	1,267	0,027	Leucine-rich repeat protein kinase family protein

Gene-ID	log2(AP vs C)	P-value	description
AT1G63040.1	1,269	0,005	a pseudogene member of the DREB subfamily A-4 of ERF/AP2 transcription factor family. The translated product contains one AP2 domain. There are 17 members in this subfamily including TINY.
AT1G32910.1	1,270	0,005	HXXXD-type acyl-transferase family protein
AT1G62980.1	1,270	0,020	expansin A18
AT1G05020.1	1,274	0,001	ENTH/ANTH/VHS superfamily protein
AT5G03210.1	1,277	0,016	unknown protein
AT1G50930.1	1,277	0,003	unknown protein
AT1G74430.1	1,280	0,013	myb domain protein 95
AT1G05800.1	1,282	0,014	alpha/beta-Hydrolases superfamily protein
AT1G73580.1	1,283	0,024	Calcium-dependent lipid-binding (CaLB domain) family protein
AT4G30430.1	1,293	0,004	tetraspanin9
AT2G46400.1	1,294	0,032	WRKY DNA-binding protein 46
AT5G47850.1	1,295	0,049	CRINKLY4 related 4
AT4G25810.1	1,296	0,003	xyloglucan endotransglycosylase 6
AT3G57450.1	1,302	0,015	unknown protein
AT4G08950.1	1,302	0,001	Phosphate-responsive 1 family protein
AT1G18740.1	1,303	0,026	Protein of unknown function (DUF793)
AT4G29640.1	1,313	0,011	Cytidine/deoxycytidylate deaminase family protein
AT1G05100.1	1,318	0,000	mitogen-activated protein kinase kinase kinase 18
AT2G23347.1	1,322	0,003	MIR844a
AT1G20520.1	1,325	0,020	Arabidopsis protein of unknown function (DUF241)
AT5G22410.1	1,325	0,047	root hair specific 18
AT1G73965.1	1,326	0,000	CLAVATA3/ESR-RELATED 13
AT2G32200.1	1,327	0,003	unknown protein
AT4G01360.1	1,329	0,006	unknown protein
AT4G37770.1	1,332	0,042	1-amino-cyclopropane-1-carboxylate synthase 8
AT2G35290.1	1,334	0,004	unknown protein
AT3G01830.1	1,339	0,003	Calcium-binding EF-hand family protein
AT5G37770.1	1,339	0,017	EF hand calcium-binding protein family
AT1G34510.1	1,339	0,014	Peroxidase superfamily protein
AT3G23170.1	1,341	0,039	unknown protein
AT3G50800.1	1,343	0,014	unknown protein
AT4G25380.1	1,349	0,005	stress-associated protein 10
AT4G18650.1	1,349	0,010	transcription factor-related
AT2G14290.1	1,361	0,019	F-box family protein with a domain of unknown function (DUF295)
AT1G56060.1	1,363	0,000	unknown protein

Gene-ID	log2(AP vs C)	P-value	description
AT5G05300.1	1,375	0,025	unknown protein
AT3G19680.1	1,383	0,002	Protein of unknown function (DUF1005)
AT1G34520.1	1,386	0,005	MBOAT (membrane bound O-acyl transferase) family protein
AT1G24140.1	1,387	0,042	Matrixin family protein
AT5G46360.1	1,390	0,021	Ca2+ activated outward rectifying K+ channel 3
AT1G67856.1	1,394	0,000	RING/U-box superfamily protein
AT5G61455.1	1,400	0,000	U2.7
AT5G57560.1	1,408	0,006	Xyloglucan endotransglucosylase/hydrolase family protein
AT4G13395.1	1,410	0,034	ROTUNDIFOLIA like 12
AT3G50060.1	1,418	0,023	myb domain protein 77
AT3G51680.1	1,427	0,002	NAD(P)-binding Rossmann-fold superfamily protein
AT1G21550.1	1,437	0,003	Calcium-binding EF-hand family protein
AT4G29780.1	1,463	0,033	unknown protein
AT3G08810.1	1,463	0,041	Galactose oxidase/kelch repeat superfamily protein
AT1G60190.1	1,470	0,001	ARM repeat superfamily protein
AT4G16820.1	1,484	0,015	alpha/beta-Hydrolases superfamily protein
AT3G60280.1	1,496	0,029	uclacyanin 3
AT5G36270.1	1,505	0,049	pseudogene of dehydroascorbate reductase
AT5G42380.1	1,505	0,038	calmodulin like 37
AT1G09950.1	1,520	0,011	RESPONSE TO ABA AND SALT 1
AT3G01730.1	1,534	0,032	unknown protein
AT5G45630.1	1,551	0,000	Protein of unknown function
AT5G05365.1	1,557	0,006	Heavy metal transport/detoxification superfamily protein
AT1G20310.1	1,559	0,001	unknown protein
AT1G77640.1	1,579	0,005	Integrase-type DNA-binding superfamily protein
AT5G59170.1	1,611	0,008	Proline-rich extensin-like family protein
AT4G18422.1	1,630	0,002	unknown protein
AT5G35110.1	1,637	0,004	unknown protein
AT2G38325.1	1,661	0,010	MIR390A
AT1G44830.1	1,673	0,032	Integrase-type DNA-binding superfamily protein
AT5G53680.1	1,695	0,003	RNA-binding (RRM/RBD/RNP motifs) family protein
AT1G21910.1	1,698	0,004	Integrase-type DNA-binding superfamily protein
AT1G56150.1	1,714	0,008	SAUR-like auxin-responsive protein family
AT2G17660.1	1,716	0,022	RPM1-interacting protein 4 (RIN4) family protein
AT3G11940.1	1,730	0,006	ribosomal protein 5A
AT4G35655.1	1,730	0,006	RPM1-interacting protein 4 (RIN4) family protein
AT1G54970.1	1,735	0,045	proline-rich protein 1

Gene-ID	log2(AP vs C)	P-value	description
AT1G70390.1	1,740	0,001	F-box and associated interaction domains-containing protein
AT5G38700.1	1,766	0,021	unknown protein
AT1G72240.1	1,772	0,015	unknown protein
AT5G22200.1	1,782	0,038	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AT5G10340.1	1,795	0,013	F-box family protein
AT1G35140.1	1,803	0,000	Phosphate-responsive 1 family protein
AT5G20740.1	1,856	0,050	Plant invertase/pectin methylesterase inhibitor superfamily protein
AT1G70380.1	1,859	0,043	F-box and associated interaction domains-containing protein
AT4G02170.1	1,861	0,000	unknown protein
AT1G18520.1	1,896	0,037	tetraspanin11
AT2G20520.1	1,904	0,029	FASCICLIN-like arabinogalactan 6
AT5G52020.1	1,993	0,001	Integrase-type DNA-binding superfamily protein
AT2G31690.1	2,125	0,021	alpha/beta-Hydrolases superfamily protein
AT1G33760.1	2,281	0,005	Integrase-type DNA-binding superfamily protein
AT1G12610.1	2,417	0,000	Integrase-type DNA-binding superfamily protein

Table A.3.: Microarray data of Arabidopsis roots treated with *A. alternatum* spore solution (2×10^7 spores/ml) and harvested 3 days after infection. Gene-ID represents genes according to annotation of The Arabidopsis Infromation resource (TAIR), LOG2 values indicate up- ($\geq$1,0) or downregulation ($\leq$1,0). Description of genes according to TAIR.

Gene-ID	log2(A vs C)	P-value	description
AT1G31580.1	-2,699	0,014	ECS1
AT1G61275.1	-2,147	0,003	U12
AT4G34035.1	-2,068	0,030	pre-tRNA
AT1G34410.1	-1,996	0,018	auxin response factor 21
AT3G01316.1	-1,696	0,015	snoRNA
AT5G27603.1	-1,612	0,008	transposable element gene
AT1G08115.1	-1,576	0,002	snRNA
AT3G06900.1	-1,565	0,014	U4.2
AT3G45140.1	-1,528	0,002	lipoxygenase 2
AT4G12470.1	-1,441	0,048	azelaic acid induced 1
AT3G57645.1	-1,419	0,002	U2.2
AT1G36530.1	-1,398	0,031	transposable element gene
AT2G15530.4	-1,350	0,021	RING/U-box superfamily protein
AT3G28945.1	-1,331	0,026	transposable element gene
AT3G55485.1	-1,314	0,047	Unknown

Gene-ID	log2(A vs C)	P-value	description
AT3G44704.1	-1,310	0,001	unknown protein
AT1G25440.1	-1,286	0,050	B-box type zinc finger protein with CCT domain
AT3G06895.1	-1,227	0,042	unknown protein
AT2G21760.1	-1,195	0,008	pre-tRNA
AT5G06165.1	-1,189	0,004	Unknown
AT1G29600.1	-1,184	0,017	Zinc finger C-x8-C-x5-C-x3-H type family protein
AT3G46190.1	-1,182	0,000	TRAF-like family protein
AT5G32410.2	-1,156	0,041	transposable element gene
AT1G64800.1	-1,153	0,013	DNA binding
AT1G15002.1	-1,144	0,047	Potential natural antisense gene
AT1G29071.1	-1,136	0,000	SNOR105 (SMALL NUCLEOLAR RNA 105)
AT1G32000.1	-1,136	0,009	unknown protein
AT5G12180.1	-1,129	0,012	calcium-dependent protein kinase 17
AT1G49230.1	-1,120	0,000	RING/U-box superfamily protein
AT4G21500.1	-1,095	0,049	unknown protein
AT5G60460.1	-1,094	0,040	Preprotein translocase Sec
AT5G16740.1	-1,091	0,023	Transmembrane amino acid transporter family protein
AT5G55270.1	-1,090	0,001	Protein of unknown function (DUF295)
AT4G14910.3	-1,083	0,024	HISTIDINE BIOSYNTHESIS 5B
AT2G05980.1	-1,082	0,038	transposable element gene
AT4G25760.1	-1,058	0,040	glutamine dumper 2
AT5G15830.1	-1,055	0,012	basic leucine-zipper 3
AT2G21480.1	-1,033	0,032	Malectin/receptor-like protein kinase family protein
AT3G48346.1	-1,033	0,016	unknown protein
AT2G28850.1	-1,027	0,010	cytochrome P450
AT3G25810.1	-1,015	0,034	Terpenoid cyclases/Protein prenyltransferases superfamily protein
AT5G05390.1	1,003	0,018	laccase 12
AT5G41750.1	1,003	0,003	Disease resistance protein (TIR-NBS-LRR class) family
AT3G11020.1	1,007	0,006	DRE/CRT-binding protein 2B
AT5G41750.2	1,008	0,004	Disease resistance protein (TIR-NBS-LRR class) family
AT1G77950.1	1,009	0,036	AGAMOUS-like 67
AT2G07667.1	1,013	0,043	unknown protein
AT2G38340.1	1,016	0,001	Integrase-type DNA-binding superfamily protein
AT3G53232.1	1,016	0,010	ROTUNDIFOLIA like 1
AT1G31290.1	1,017	0,031	ARGONAUTE 3
AT5G40000.1	1,017	0,008	P-loop containing nucleoside triphosphate hydrolases superfamily protein

Gene-ID	log2(A vs C)	P-value	description
AT4G21926.1	1,019	0,002	unknown protein
AT2G36295.1	1,019	0,004	unknown protein
AT1G52855.1	1,037	0,043	unknown protein
AT5G46960.1	1,042	0,047	Plant invertase/pectin methylesterase inhibitor superfamily protein
AT2G35290.1	1,043	0,001	unknown protein
AT4G33070.1	1,056	0,031	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein
AT1G17345.1	1,076	0,032	SAUR-like auxin-responsive protein family
AT5G60910.1	1,077	0,043	AGAMOUS-like 8
AT2G24320.1	1,079	0,048	alpha/beta-Hydrolases superfamily protein
AT1G43640.1	1,079	0,032	tubby like protein 5
AT1G60190.1	1,079	0,022	ARM repeat superfamily protein
AT2G32020.1	1,083	0,046	Acyl-CoA N-acyltransferases (NAT) superfamily protein
AT1G10990.2	1,086	0,016	unknown protein
AT1G09950.1	1,088	0,006	RESPONSE TO ABA AND SALT 1
AT1G65481.1	1,100	0,000	unknown protein
AT2G02320.1	1,102	0,013	phloem protein 2-B7
AT1G61630.1	1,109	0,022	equilibrative nucleoside transporter 7
AT2G34390.1	1,113	0,016	NOD26-like intrinsic protein 2
AT1G04370.1	1,120	0,000	Ethylene-responsive element binding factor 14
AT5G51440.1	1,123	0,007	HSP20-like chaperones superfamily protein
AT3G19680.1	1,131	0,025	Protein of unknown function (DUF1005)
AT1G07890.8	1,132	0,023	ascorbate peroxidase 1
AT1G73010.1	1,136	0,001	phosphate starvation-induced gene 2
AT1G21550.1	1,137	0,005	Calcium-binding EF-hand family protein
AT5G14090.1	1,139	0,043	unknown protein
AT4G13480.1	1,141	0,006	myb domain protein 79
AT3G17690.1	1,141	0,025	cyclic nucleotide gated channel 19
AT1G52857.1	1,148	0,025	unknown protein
AT4G39290.1	1,148	0,046	Galactose oxidase/kelch repeat superfamily protein
AT1G67856.1	1,151	0,000	RING/U-box superfamily protein
AT1G65680.1	1,151	0,025	expansin B2
AT5G59170.1	1,154	0,036	Proline-rich extensin-like family protein
AT4G24110.1	1,156	0,008	unknown protein
AT5G18020.1	1,156	0,012	SAUR-like auxin-responsive protein family
AT1G73120.1	1,159	0,001	unknown protein
AT1G18290.1	1,160	0,010	unknown protein
AT2G30760.1	1,162	0,032	unknown protein

Gene-ID	log2(A vs C)	P-value	description
AT5G12020.1	1,166	0,007	17.6 kDa class II heat shock protein
AT5G59330.1	1,173	0,005	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT3G01830.1	1,175	0,018	Calcium-binding EF-hand family protein
AT2G44070.1	1,178	0,013	NagB/RpiA/CoA transferase-like superfamily protein
AT5G54165.1	1,187	0,011	unknown protein
AT4G37240.1	1,190	0,003	unknown protein
AT3G47480.1	1,194	0,035	Calcium-binding EF-hand family protein
AT1G43800.1	1,203	0,003	Plant stearoyl-acyl-carrier-protein desaturase family protein
AT4G33930.1	1,210	0,025	Cupredoxin superfamily protein
AT4G08950.1	1,212	0,015	Phosphate-responsive 1 family protein
AT4G25200.1	1,213	0,016	mitochondrion-localized small heat shock protein 23.6
AT3G56980.1	1,215	0,009	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT1G07400.1	1,218	0,010	HSP20-like chaperones superfamily protein
AT3G48640.1	1,219	0,038	unknown protein
AT1G18520.1	1,223	0,044	tetraspanin11
AT1G52820.1	1,227	0,003	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AT5G01100.1	1,232	0,008	O-fucosyltransferase family protein
AT3G29375.2	1,233	0,045	XH domain-containing protein
AT3G22830.1	1,252	0,005	heat shock transcription factor A6B
AT5G45630.1	1,256	0,011	Protein of unknown function
AT4G25810.1	1,260	0,001	xyloglucan endotransglycosylase 6
AT1G52565.1	1,265	0,000	unknown protein
AT3G51680.1	1,285	0,000	NAD(P)-binding Rossmann-fold superfamily protein
AT1G47395.1	1,288	0,021	unknown protein
AT5G20740.1	1,307	0,004	Plant invertase/pectin methylesterase inhibitor superfamily protein
AT5G05220.1	1,307	0,021	unknown protein
AT2G03822.1	1,314	0,020	unknown protein
AT2G17850.1	1,315	0,017	Rhodanese/Cell cycle control phosphatase superfamily protein
AT2G02340.1	1,323	0,019	phloem protein 2-B8
AT1G53060.1	1,324	0,050	Legume lectin family protein
AT2G36780.1	1,325	0,008	UDP-Glycosyltransferase superfamily protein
AT1G47400.1	1,329	0,017	unknown protein
AT5G52350.1	1,344	0,027	exocyst subunit exo70 family protein A3
AT2G32200.1	1,352	0,043	unknown protein
AT3G21720.1	1,353	0,024	isocitrate lyase
AT4G06523.1	1,363	0,049	transposable element gene

Gene-ID	log2(A vs C)	P-value	description
AT5G61455.1	1,370	0,002	U2.7
AT1G05100.1	1,371	0,003	mitogen-activated protein kinase kinase kinase 18
AT1G47410.2	1,375	0,012	unknown protein
AT5G12030.1	1,379	0,008	heat shock protein 17.6A
AT5G64450.1	1,385	0,018	BEST Arabidopsis thaliana protein match is: Putative endonuclease or glycosyl hydrolase (TAIR:AT3G62200.1)
AT5G53680.1	1,385	0,013	RNA-binding (RRM/RBD/RNP motifs) family protein
AT1G56060.1	1,388	0,018	unknown protein
AT2G41340.1	1,391	0,001	RNA polymerase II fifth largest subunit
AT3G06962.1	1,391	0,043	other RNA
AT5G51480.1	1,443	0,008	SKU5 similar 2
AT1G08860.1	1,446	0,001	Calcium-dependent phospholipid-binding Copine family protein
AT3G23220.1	1,462	0,040	Integrase-type DNA-binding superfamily protein
AT3G26960.1	1,465	0,046	Pollen Ole e 1 allergen and extensin family protein
AT5G05365.1	1,466	0,001	Heavy metal transport/detoxification superfamily protein
AT1G51030.1	1,471	0,001	unknown protein
AT1G51010.1	1,491	0,003	unknown protein
AT4G28160.1	1,495	0,008	hydroxyproline-rich glycoprotein family protein
AT4G18422.1	1,505	0,020	unknown protein
AT2G17660.1	1,505	0,002	RPM1-interacting protein 4 (RIN4) family protein
AT5G14650.1	1,506	0,004	Pectin lyase-like superfamily protein
AT5G64060.1	1,507	0,014	NAC domain containing protein 103
AT4G30430.1	1,527	0,015	tetraspanin9
AT4G35655.1	1,529	0,000	RPM1-interacting protein 4 (RIN4) family protein
AT3G24510.1	1,560	0,036	Defensin-like (DEFL) family protein
AT4G39360.1	1,563	0,006	unknown protein
AT1G74670.1	1,620	0,014	Gibberellin-regulated family protein
AT5G41900.1	1,622	0,018	alpha/beta-Hydrolases superfamily protein
AT5G52020.1	1,626	0,000	Integrase-type DNA-binding superfamily protein
AT4G28420.2	1,679	0,021	Tyrosine transaminase family protein
AT5G28821.1	1,684	0,004	unknown protein
AT4G01360.1	1,687	0,000	unknown protein
AT5G44430.1	1,733	0,011	plant defensin 1.2C
AT1G51020.1	1,746	0,009	unknown protein
AT2G36770.1	1,793	0,024	UDP-Glycosyltransferase superfamily protein
AT2G47520.1	1,794	0,000	Integrase-type DNA-binding superfamily protein
AT1G67100.1	1,807	0,020	LOB domain-containing protein 40

Gene-ID	log2(A vs C)	P-value	description
AT1G21910.1	1,808	0,050	Integrase-type DNA-binding superfamily protein
AT1G71000.1	1,826	0,035	Chaperone DnaJ-domain superfamily protein
AT4G29305.1	1,829	0,001	low-molecular-weight cysteine-rich 25
AT2G44460.1	1,851	0,030	beta glucosidase 28
AT1G35140.1	1,859	0,011	Phosphate-responsive 1 family protein
AT5G66890.1	1,929	0,001	Leucine-rich repeat (LRR) family protein
AT1G75830.1	1,938	0,000	low-molecular-weight cysteine-rich 67
AT2G14247.1	1,940	0,011	Expressed protein
AT3G30725.1	1,994	0,036	glutamine dumper 6
AT3G56210.1	1,995	0,008	ARM repeat superfamily protein
AT5G43650.1	1,999	0,036	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT3G29970.1	2,024	0,036	B12D protein
AT2G23347.1	2,032	0,000	MIR844a
AT5G19880.1	2,033	0,003	Peroxidase superfamily protein
AT1G74430.1	2,080	0,000	myb domain protein 95
AT4G02170.1	2,107	0,033	unknown protein
AT3G47720.1	2,137	0,008	similar to RCD one 4
AT5G10040.1	2,162	0,005	unknown protein
AT1G12610.1	2,376	0,008	Integrase-type DNA-binding superfamily protein
AT2G30766.1	3,859	0,000	unknown protein

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A.2. Unabhängigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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